PHYSIOLOGICAL, MORPHOLOGICAL, AND BEHAVIOURAL EFFECTS OF DEVELOPMENTAL EXPOSURE TO AROCLOR 1254 IN NESTLING AND JUVENILE SONGBIRDS

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ABSTRACT

Over the past several decades, there has been growing concern among the public and scientific community regarding adverse health effects resulting from exposure to natural and synthetic compounds that act as endocrine disrupters. The structural similarity of many of these compounds to natural hormones and receptors, as well as their ubiquity in the environment, can result in the potential for interference with the endocrine system of wildlife and humans. Much of the research examining the adverse effects of wildlife exposure to endocrine disrupting chemicals (EDCs) has focused on effects on reproduction or short-term changes in hormone physiology. However, organisms exposed to low levels of EDCs at early life stages could also be susceptible to developmental effects, including neurological and other physiological changes affecting later life stages. In birds, migration can be an important component of the annual life cycle and it can be vulnerable to disruption given that it is under endogenous hormonal and neurological control. Previous studies have shown that developmental exposure of birds to thyroid hormone disruptors, such as polychlorinated biphenyls (PCBs), have resulted in reduced hatching success, lower growth rates, teratogenicity, impaired development, and immunotoxicity. In this thesis, I aimed to supplement what is currently known regarding the effects of developmental exposure to low levels of a mixture of endocrine disrupting chemicals in songbirds, as well as further investigate the latent consequences of such an exposure on migratory life stages.

I initially investigated the potential physiological and developmental effects of early exposure to Aroclor 1254, a PCB mixture, in two passerine songbird species: European starlings (*Sturnus vulgaris*) and red-winged blackbirds (*Agelaius phoeniceus*) during the critical nestling period. In 2011, European starlings and red-winged blackbirds were orally administered Aroclor

1254 over the period of nestling development, which was repeated in 2012 with only European starlings. For both years, morphological parameters (body mass, tarsus, wing-chord and bill-lengths) were measured daily and plasma thyroid hormones were measured. Additional measurements of wing chord and tarsus length fluctuating asymmetry (FA) were taken in the second year, to further assess contaminant-induced alterations in developmental stability. I found that treatment with environmentally-relevant levels of Aroclor 1254 caused increasing liver residues above the controls but did not result in overt effects on morphological growth parameters during the nestling period in either starlings or red-winged blackbirds. However, we did observe significant differences in 2012 starling's wing chord FA at day 10 and 13, and tarsus length FA between all treatment groups and controls, indicating the potential for PCB-induced stress. Nestling thyroid hormone profiles (T3) sampled throughout the nestling period supported developmental changes but did not reveal any differences among treatment groups.

Starlings were subsequently reared in captivity and further tested during a simulated autumn migration. Migratory activity and orientation were tested using Emlen funnel trials over 6 consecutive weeks. Across treatment groups, we found a significant increase in mass, fat, and feather moult, and decreasing plasma thyroid hormones over time. At 12L:12D, control birds showed a peak in activity and a directional preference for 155.95° (South-southeast), while high-dosed birds did not. High-dosed birds showed a delayed directional preference for 197.48° (South-southwest) under 10L:14D, concomitant with apparent delays in moult. These findings link alterations in avian migratory behaviour to contaminant-specific mechanisms. Exposure to a ubiquitous environmental endocrine disruptor exerted only subtle short-term effects during the period of exposure but importantly, latent effects may be far more relevant for individual fitness. We discuss how the impacts of exposure during early stages of development were not significant

for short-term nest success, but can still give rise to longer time-scale effects that are potentially relevant for survival and population stability for migratory birds.

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PREFACE

Chapter 1 of this thesis is a general introduction and Chapters 2 and 3 are organized as manuscripts for publication in scientific journals. Thus, there is some repetition of introductions, materials, and methods throughout each chapter. Chapter 2 is intended for later submission for publication and Chapter 3 has been submitted for publication to *Environmental Science and Technology*. Chapter 4 is a conclusion to both studies, with recommendations for future research.

1 CHAPTER 1: GENERAL INTRODUCTION

1.1 Endocrine disrupting chemicals in the environment

There is an increasing level of both scientific and public concern regarding endocrine disruption by natural and synthetic chemical pollutants that are widespread in the environment and are commonly found in the tissues of wildlife and humans[1, 2]. Endocrine disrupting chemicals (EDCs) are chemicals that include a diverse array of exogenous compounds that are hormonally active or can mimic the action of endogenous hormones, giving rise to the potential for interference with homeostasis, reproduction, development, and/or behaviour[3-5]. EDCs found in the environment include a wide variety of chemicals and complex mixtures, making it difficult to elucidate common mechanisms of action on target tissues and organs[1].

1.2 Polychlorinated biphenyls (PCBs): Structure, properties and mechanisms of action

One such group of structurally similar and environmentally pervasive chemicals, the polycyclic halogenated aromatic hydrocarbons (PHAHs), is comprised mainly of brominated biphenyls, naphthalenes, azoxybenzenes, polychlorinated dibenzo-*p*-dioxins (PCDDs), polychlorinated dibenzofurans (PCDFs), and polychlorinated biphenyls (PCBs)[6]. This family of contaminants includes the most toxic halogenated aromatic compound, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), which is used as the reference compound when determining the relative toxicities (toxic equivalency factors, TEFs) of other dioxins and dioxin-like compounds (DLCs). 2,3,7,8-TCDD is the most potent of inducer of aryl hydrocarbon hydroxylase (AHH)[7] activity, which is controlled by the aryl hydrocarbon receptor (AhR), a

ligand-activated nuclear transcription factor[8]. Non-*ortho*-substituted PCBs and certain mono-*ortho*-substituted PCBs have the potential to adopt a planar configuration and bind to the cytoplasmic AhR[9, 10]. The suite of clinical symptoms observed in humans and laboratory animals resulting from exposure to PCBs, PCDFs, and PCDDs signify that these chemicals most likely act via a common mechanism of action[9, 10], which will be discussed further in Section 1.3. Dioxins and DLCs bind to and activate the AhR, which is now thought to be the main pathway by which these compounds mediate their toxic effects, which manifest in animals as carcinogenicity, embryotoxicity, immunotoxicity, and teratogenicity[6, 10].

1.2.1 Polychlorinated biphenyls – Global sources, uses, and structural properties

One complex group of EDCs that are commonly of concern, due in part to a wide array of adverse effects documented in laboratory and wildlife species, are PCBs[11]. PCBs are synthetic organic chemicals that were prepared as complex mixtures of congeners with varying degrees of chlorination. It has been estimated that over one million tons of PCBs were produced worldwide between 1930 and 1993[12]. In the United States, PCBs were previously manufactured and marketed as commercial Aroclor mixtures (e.g. Aroclor 1248, 1254, 1260) by the Monsanto Chemical Company, containing chlorine contents of 21, 32, 42, 54, 60, and 62% by weight[13, 14]. A PCB congener refers to the total number of chlorines attached to the two biphenyl rings, as well as their specific position; therefore, there are 209 different PCB congeners and ten different congener classes[9]. Other manufacturers worldwide produced similar commercial mixtures of PCBs, under trade names such as Clophen® (Germany), Kanechlor® (Japan), Fenclor® (Italy), Phenoclor® and Pyralene® (France), Sovol® (the former U.S.S.R), Chlorfen® (Poland), and Delor® (the former Czechoslovakia)[13, 14].

PCBs were suitable for a wide range of industrial applications due to their high thermal and chemical stability, high lipophilicity, low volatility, and low solubility in water[13, 17], leading to their use as lubricants, hydraulic fluids, electrical insulators, pesticide extenders, adhesives, flame retardants, and plasticizers[14, 16, 18]. PCBs are marginally soluble in water and exhibit slow degradation[9, 19]. Improper waste management, disposal strategies, and leakage are some of the factors contributing to the continued release of PCBs into the environment[14] and it has been estimated that 31% of the world's total production of PCBs has been released into the environment[16]. Consequently, PCBs occur at detectable concentrations in virtually every level of the global ecosystem[2]. The composition of commercial PCB mixtures is dissimilar to the composition of PCBs in environmental media, which arises from differing physicochemical properties, degradation rates, and mixing within environmental compartments[14, 20], resulting in the need for studies of the toxic effects of environmental PCB mixtures[14]. While the intentional production of PCBs ceased in 1977 by Monsanto Co.[13], other countries continued their manufacture until the late 1980s. The persistence and lipophilic properties of PCBs facilitate bioaccumulation and biomagnification through food chains[2], resulting in PCBs being one of the most pervasive and widespread groups of environmental contaminants[21, 22].

Many of the more toxic effects are mediated by non-*ortho*-substituted PCBs with a coplanar configuration[20, 23], which have been suggested to have higher AhR binding affinities[23] and a greater effect on modifying cognition in humans[15]. As mentioned above, Aroclor 1254 is a complex mixture of PCB congeners that varies between batches and is approximately 54% chlorine by weight[15, 16]; it is primarily composed of tetra-, penta- and hexa-chloro-biphenyls, as determined using chromatographic techniques[14]. In birds, it has been noted that the most severe

effects result from exposure to those Aroclor mixtures that were moderately chlorinated, Aroclor 1254 being one of those mixtures[16].

1.3 Mechanisms of action: PCBs and the thyroid hormone system

The toxicity of PCBs could be due in part to their biotransformation by the cytochrome P450 monooxygenase enzyme system, producing hydroxylated PCB metabolites (OH-PCBs). PCBs and their metabolites are structurally analogous to endogenous hormones and can mediate their toxic effects by acting as either agonists or antagonists with estrogen, androgen, and/or glucocorticoid receptors[24-26], activation of AhR-mediated pathways[27, 28], interference with calcium homeostasis[29], and/or through interactions with thyroid hormone and vitamin A transport systems[30, 31]. The structural similarity of PCBs and OH-PCBs to endogenous hormones can give rise to the potential for the disruption of steroid responsive pathways, such as sexual differentiation of the song system[32], maturation, and control of adult breeding[33] in birds.

Exposure of experimental animals and wildlife to PCBs has resulted in a wide range of effects on thyroid function[34, 35], with consequences for thyroid gland morphology and function, thyroid hormone synthesis, secretion, metabolism, transport, binding, and excretion[30, 36]. Results of these studies have led to a rapid rise in wildlife studies reporting thyroid disfunction as an indicator of environmental contamination[34]. Laboratory and field studies of birds have reported reduced hatching success, reduced nest attentiveness, altered parental behaviour, altered enzymatic activity, lower growth rates, teratogenicity, carcinogenicity, and immunotoxicity[33, 37-39]. The structural similarity of PCBs and their toxic metabolites to thyroid hormones have led to wide ranging effects on processes common between humans and experimental animals, such as impairments in learning, cognitive abilities, memory, and behaviour. The occurrence of PCBs at

detectable concentrations in a wide range of avian species[17, 40] may contribute to disruption of thyroid-dependent processes in avian wildlife populations. PCB congeners and OH-PCBs can interfere with the thyroid hormone and vitamin A transport system[30, 31] through competitive inhibition of thyroxine (T4) binding to transthyretin (TTR), its transport protein[31]. By displacing T4 from TTR, free T4 in the blood can increase, resulting in heightened T4 metabolism and excretion, which may eventually result in hypothyroidism due to a sustained decrease in circulating T4 levels[19, 30]. As a result, this interference may have consequences for: (1) thyroid hormone metabolism, (2) thyroid gland morphology and function and (3) for levels of hormone transport binding proteins[30]. Much of what is known about the mechanisms through which PCBs interfere with thyroid function has been elucidated through laboratory studies with mammals, which explain much of the first level of interaction. It is important to note that the PCB-induced alterations observed in both laboratory and wildlife species are the result of a number of interactions and may not be attributed to one single mechanism.

Many of the alterations in thyroid hormone metabolism may also in part be initiated by binding of PCBs to the AhR protein[7]. There are a number of AhRs in mammals; however, only two forms of AhR in birds have been identified, AhR1 and AhR2[8, 41]. These proteins contribute to the relative sensitivity of bird species to dioxins and DLCs, which has been found to vary by more than 1000-fold. The sensitivity of bird species to DLCs can be predicted by the amino acid sequence of the AhR1 ligand binding domain (LBD), specifically the identity of amino acids at locations 324 and 380. The identities of these amino acids allows for the classification of birds into three groups, based on differential levels of sensitivity (low, moderate, and high sensitivity)[42]. Binding of PCBs to AhR is followed by an activation step and the cytosol-receptor complexes are translocated to the nucleus, where they form nucleus-receptor complexes that act

to stimulate cytochrome P-4501A1 (CYP1A1) gene transcription through interactions with specific DNA sequences located upstream of the CYP450 gene[9, 42]. It has been shown that the most well characterized response upon exposure to PCBs is the induction of both phase I and II biotransformation enzyme systems in laboratory animals; specifically CYP1A1 and cytochrome P-4501A2 (CYP1A2) proteins and their associated monooxygenase activities of AHH and ethoxyresorufin O-deethylase (EROD). The phase II enzymes that are mainly induced by halogenated hydrocarbons are glutathione s-transferases and glucuronosyl transferases. Uridine diphosphate glucuronosyltransferase (UDP-GT) conjugates T4, the main product of the thyroid gland, into T4-glucuronide, which is then excreted in the bile[6, 9, 23, 43]. PCB-induced induction of UDP-GT can then result in decreased free circulating levels of T4 as glucuronidation and subsequent excretion in the bile increases[43]. Sprague-Dawley rats fed low doses of PCB 126 and PCB 156 over thirteen weeks showed clear dose dependent increases in UGT1A1, T4UGT and CYP1A1 enzyme activities, along with a decrease in total and free thyroxine hormone levels. This decrease in plasma thyroid hormone levels is significantly correlated with the increase of T4UGT enzyme activity after PCB administration[44]. It has been shown that the AhR controls the expression of AHH activity, which is mainly catalyzed by CYP1A1 and so these results, along with the co-induction of hepatic CYP1A1 and UGT1A1 enzymes, demonstrate that there may be an Ah-receptor mediated process involved in the glucuronidation of T4 and the consequent decrease in circulating concentrations of T4[7].

1.4 Thyroid hormone function

The follicles of the thyroid gland produce thyroglobulin, a globular glycoprotein, containing iodinated tyrosine residues, which are linked to produce monoiodotyrosine, diiodotyrosine and the active thyroid hormones triiodothyronine (T3) and T4[45]. In birds, thyroid

hormones are essential for controlling basal metabolic rate, pre-and postnatal differentiation and central nervous system development, and also for regulating body weight, muscle and bone growth, initiation of moult and plumage growth, lipid metabolism, and secondary sex characteristics[45, 46]. They are also vitally important for nervous system and brain development[36, 47], controlling neuronal production, migration, and differentiation, myelination, and synaptogenesis[47]. The effects of PCBs and other dioxin-like compounds on brain development (e.g. asymmetry) in birds have been characterized[19], which could have negative consequences for migratory ability later in life.

Altricial bird species are born without the capacity to thermoregulate and have very low plasma thyroid hormone concentrations. The development of endothermy in nestling altricial species, such as red-winged blackbirds (*Agelaius phoeniceus*), European starlings (*Sturnus vulgaris*), ring doves (*Streptopelia risoria),* and great tits (*Parus major*), relies on the functional maturation of the thyroid gland, which is reflected by the increase in plasma T3 and T4 levels[48]. At hatch, red-winged blackbirds have low plasma T3 and T4 concentrations, which steadily increase over the posthatch period of 10-12 days, to reach plasma concentrations similar to that of adults[49]. In European starlings, both plasma T3 and T4 concentrations increase up until day 5 posthatch, after which only T4 continues to increase during development up until day 15, when levels are comparable to adult starlings[50]. The nestling period for altricial bird species appears to be when critical development of thyroid function and consequent endothermic capabilities occurs[49], making the period of nestling development the most critical time for measuring changes in thyroid hormones brought on by exposure to environmental contaminants. Also, the risk of exposure and sensitivity to PCBs and OH-PCBs during the critical window of nestling development is likely greater than that of adults because young individuals undergo large and

rapid structural and functional changes, making them particularly vulnerable to any adverse effects that may precipitate later in life[51, 52]. Altricial and precocial birds express different states of development at hatch and ontogenetic patterns of growth[53-56], which partly influences the most sensitive period for EDC exposure[51]. Disruptions in early neural development, gonadal differentiation, and sex steroid hormone production resulting from early exposure to EDCs are often permanent[5] and can have subsequent impacts on later biological processes, particularly in altricial species[51].

1.4.1 Regulation of migration in birds

Annual life cycles in most birds consist of breeding, moult, spring and autumn migrations, which are all energetically expensive physiological processes. The timing and duration of each stage is controlled by the bird and in most cases, there is minimal temporal overlap between stages[57, 58], to optimize the success of each life history stage. Regulation of the timing and manifestation of avian reproduction, moult, and migration in many passerine species is controlled by multiple environmental inputs, primarily seasonal changes in photoperiod, and intrinsic factors such as endogenous circadian and circannual rhythms within the bird[57, 59]. Reproduction and moult are primarily regulated by seasonal changes in daylength in birds from temperate and high latitudes, whereas tropical migrants rely more on endogenous components[60].

Neural, neuroendocrine, and endocrine pathways are directly responsible for the regulation of the morphological, physiological, and behavioural characteristics that are expressed in response to seasonal changes in the environment, such as photoperiod[61, 62]. Physiological and behavioural changes associated with vernal and autumn migrations, such as hyperphagia, premigratory fattening, *Zugunruhe*, moult, and energy usage, are regulated by different aspects

of the endocrine system[63]. Autumnal migration is thought to be regulated by a number of hormones, such as thyroid hormones, prolactin and glucocorticoids, while gonadal steroids such as testosterone, are thought to be of primary importance for vernal (spring) migration[64,65].

Activity of the thyroid gland is dependent on photoperiod[45]. Avian migration is characterized by two distinct periods: (i) the premigratory period in which the bird prepares for flight by laying down deposits of fat and (ii) the actual period of flight toward either summer or wintering grounds, during which birds will encounter new habitats and ecological barriers, resulting in burdens on their energy and water budgets[46]. Birds will alternate between periods of hyperphagia, in which fat and protein reserves will quickly be replenished, and periods of energy consumption during flight[66]. Accompanying the premigratory period is the phenomenon known as *Zugunruhe*, or migratory restlessness, which is typically assessed by hopping activity frequency[46, 67, 68]. Pathak & Chandola (1982)[68] demonstrated that when the thyroid gland of captive red-headed buntings (*Emberiza bruniceps*) was removed, this period of restlessness was absent and resumed upon administration of T3 and T4. In these same thyroidectomized birds, no premigratory fattening period took place. The absence of the premigratory fattening and restlessness was also reversed upon replacement with either T3 or T4, indicating that specific levels of these hormones play an essential role in initiation of migration. This absence of the thyroid gland was also associated with the suppression of gonadal growth and feather growth during moult, which was reversed when T4 was replaced, indicating that higher circulating levels of T4 are perhaps more important during these physiological events[46, 68]. These observations are further supported by studies which indicate that high circulating levels of T4 during the fall premigratory period in the Canada goose (*Branta canadensis interior*) were associated with moult. It is important to note that both T3 and T4 are important for the entire complement of

events associated with migration and it is thought that both are biologically active and equipotent forms[70], indicating that thyroid T4 and plasma T3 and T4 concentrations throughout important life history events of a bird are likely instrumental for determining effects of environmental contaminants on the thyroid hormone system.

1.5 Techniques for recording migratory behaviour

When held in captivity during migratory season, the *Zugunruhe* exhibited by passerines is the main measure of assessing migratory activity[46, 67]. Birds in migratory condition had historically been placed in circular cages and their orientation activities recorded visually, or with microswitches and electronic counters. Emlen *et al*. (1966)[67] introduced the Emlen funnel, which has largely been the instrument of choice for orientation experiments since the 1960s[71]. Emlen funnels gave researchers the ability to obtain results using a larger number of birds, eliminating the need for long periods of observation and reducing the associated costs. The circular funnels were lined with white blotting paper, with an ink pad on the floor of the funnel and covered with mesh screens; blocking everything but the sky from the view of the bird being tested. Birds in migratory condition will exhibit behaviour such as quivering and wing-spreading, and will hop onto the paper, leaving inked footprints in the direction of preferred flight. The accumulation of footprints on the paper is then the record of the bird's migratory activity[67]. A large drawback of this technique was that the birds would become covered in ink and so inkpads were eventually replaced by typewriter correction paper and then most recently, thermal paper (fax paper). Friction is generated between the paper and the bird's claws when they move within the funnel and the paper irreversibly changes colour from white to blue, generating a record of migratory activity and preferred orientation. Other methods include the use of cage/funnel microswitches or computer controlled Emlen funnels with emitters and sensors of infrared

light[72]. While there are advantages associated with the use of thermal paper in funnels, limitations include subjectivity in counting scratch marks, little to no temporal information, environmental factors affecting sensitivity of paper, and birds having a mass too low to leave a scratch mark[71]. Similar limitations negatively affect the suitability of using microswitches, such as humidity lowering their sensitivity, lack of additional behavioural observations, and low resolution of data[71]. The limitations of these techniques for recording orientation and migratory restlessness led to our development of a modified Emlen funnel, used in conjunction with multiple video-tracking apparatuses to record the movement of a large number of funnels at once. This, combined with the use of the newly created video-tracking program, BirdOriTrack[71], allowed for inexpensive and objective analysis of the orientation and activity of birds in migratory condition.

1.6 Study species: testing a model for migratory studies

While there have been a large number of studies conducted using Emlen funnels to examine migratory behaviour of songbirds[72], these studies primarily use nocturnal migrants and/or birds that are protected under some form of migratory protective legislation, thereby limiting sufficient sample sizes for behavioural studies. It was therefore necessary to address these limitations and to develop a suitable model for testing the effects of environmental contaminants on a migratory test species. Two songbird species, the European starling (*S. vulgaris*) and the red-winged blackbird (*A. phoeniceus*) were selected for the studies, due to high ubiquity throughout the study area, migratory life histories, intelligence, and robustness in captivity. Both species are considered agricultural pest species and so are not protected under the *Migratory Birds Convention Act, 1994*[73], thus requiring no federal permits for handling and collection.

The European starling is a widely distributed non-native passerine songbird that was introduced into North America in 1891[74]. Breeding starlings readily adopt nestboxes and are diurnal partial migrants, with a well-studied breeding and migratory ecology[22, 75]. Red-winged blackbirds are also a ubiquitous and well-studied passerine species, with a partial migration strategy[76]. They construct semi-conspicuous nests at high densities in wetland areas[77], making it relatively easy to find a sufficient sample size of nests. These characteristics, combined with the altricial development pattern of both species[78], make them highly suitable for examining morphological, physiological, and behavioural changes resulting from developmental exposure to PCBs[79].

Both starlings and red-winged blackbirds undergo autumn and spring migration at these mid-latitudes of the Canadian Prairies, making them suitable for studies of migratory orientation. In North America, autumn migration of starlings normally occurs from late-September to mid-October and the spring migration from mid-February to early April[75]. Red-winged blackbirds begin their fall migration during September to October and their spring migration takes place from mid-February to mid-May[80]. However, these are generalizations and the times can vary with geographic locality and weather. Starlings are established as permanent residents throughout their breeding ranges of North America, the British Isles, and continental Europe[75]. However, banding data demonstrates some northern populations of starlings are migratory.

1.7 Objectives

The specific research objectives of my thesis were:

- Investigate the potential effects of a developmental exposure to Aroclor 1254, a PCB mixture and known endocrine-disrupting compound, on liver residues, thyroid hormones and morphology in 2 nestling songbird species, the European starling (*Sturnus vulgaris*) and the red-winged blackbird (*Agelaius phoeniceus*) (Chapter 2).

- Evaluate any latent effects of nestling exposure to Aroclor 1254 on moult, fattening, migratory activity, and orientation behaviour in captive European starlings under a simulated autumn migration. (Chapter 3).

2 CHAPTER 2

Assessment of changes in liver residues, thyroid hormones and morphology in nestling songbirds exposed to Aroclor 1254 during early development

Study contributions

The design of the 2011 and 2012 nestling dosing studies, conduction of field work, captive work, laboratory work, primary data analysis, and writing of manuscript was conducted by Leanne M. Flahr. Christy Morrissey, Principle Investigator, was also directly involved in conceiving and designing the studies, provided training of first author, funding, assistance both in the field and captivity, and editing of the manuscript. Nicole Michel provided guidance and assistance with the statistical models and data analysis. Alexander Zahara acted as a field assistant with the entire 2012 field and captive studies. Garry Codling provided assistance in GC/MS analysis of dosing solution and liver residue Aroclor 1254 concentrations, and with analysis of final data output. Paul Jones provided assistance with modification of method adapted from EPA Method 1668B (US-EPA 2008) for GC/MS analysis of dosing solution and liver residue Aroclor 1254 concentrations.

The study reported here was approved by the University of Saskatchewan's Animal Research Ethics Board and adheres to the Canadian Council on Animal Care guidelines for humane animal use and the University of Saskatchewan Policy on Care and Use of Animals in Research (Animal Use Protocol #20110043).

2.1 Abstract

There is an increasing level of concern regarding the consequences of early exposure to endocrine disrupting chemical pollutants that are increasingly widespread in the environment. In avian models, thyroid hormone disruptors such as polychlorinated biphenyls (PCBs) can result in reduced hatching success, lower growth rates, teratogenicity, impaired development, and immunotoxicity, which can have fitness consequences persisting into adulthood. We investigated the potential developmental effects of early exposure to Aroclor 1254, a PCB mixture, in two songbird species: European starlings (*Sturnus vulgaris*) and red-winged blackbirds (*Agelaius phoeniceus*) during the critical nestling period. As part of a pilot study in 2011, European starlings and red-winged blackbirds were orally administered 0, 0.35, or 0.70 µg Aroclor 1254/g-bw/day over the period of nestling development. In 2012, starlings were orally administered 0, 0.35, 0.70, or 1.05 µg Aroclor 1254/g-bw/day from 1 through 18 days-post-hatch. Body mass, tarsus, wing-chord and bill-lengths were measured daily and blood was taken to measure circulating plasma thyroid hormones. Subsets of nestlings were sacrificed as fledglings, and liver samples collected for contaminant analyses. Mean Aroclor 1254 liver residues were greater ($p < 0.001$) in 2012 treated starlings when compared to controls, with no significant differences found in liver residues between treatments in either 2011 starlings or red-winged blackbirds. Treatment with environmentally-relevant levels of Aroclor 1254 during the nestling period did not significantly alter survival or overt morphological growth variables at any time point. Though, an increase in fluctuating asymmetry of wing at day 10 and 13 and overall tarsus length signalled PCB-induced stress relative to controls which did not persist over time through fledging. Nestling thyroid hormone profiles (T3) sampled three times over the

nestling period paralleled developmental changes but did not reveal any differences among dose groups. This study demonstrates that low-level exposure to a ubiquitous environmental endocrine disruptor exerts only subtle short-term effects during the period of exposure but importantly, permanent latent effects may be far more relevant for individual fitness.

2.2 Introduction

There is substantial evidence documenting the adverse health effects in laboratory animals, wildlife species, and humans resulting from exposure to environmental contaminants that interact with the endocrine system[81] . Endocrine disrupting chemicals (EDCs) are a group of natural and synthetic chemicals that can mimic the action of endogenous hormones and have the potential to interfere with hormonal and homeostatic systems of organisms[3-5, 82], resulting in adverse effects on reproductive, immune, and/or neurological systems[32, 83]. EDCs are ubiquitous in the environment and include a wide variety of chemicals and complex mixtures, making it difficult to elucidate common mechanisms of action on target tissues and organs[1, 84, 85].

One such complex group of EDCs that are of particular scientific and public concern, due in part to their ubiquity, persistence, lipophilicity, and the wide array of adverse effects documented in laboratory and wildlife species, are polychlorinated biphenyls (PCBs)[11, 86]. PCBs are synthetic organic contaminants that were synthesized as mixtures of isomers with varying degrees of chlorination[2] and previously manufactured and marketed as commercial Aroclor mixtures (e.g Aroclor 1248, 1254, 1260)[2, 13, 15, 29]. PCBs and their hydroxylated metabolites (OH-PCBs), are structurally analogous to endogenous hormones and can mediate their effects through activation of aryl hydrocarbon receptor (AhR) - mediated pathways[27, 28], acting as either agonists or antagonists with estrogen, androgen, and/or glucocorticoid receptors[24-26], interference with

calcium homeostasis[29], and/or through interactions with thyroid hormone and vitamin A transport systems[30, 31]. Laboratory and field studies of birds have reported reduced hatching success, reduced nest attentiveness, altered parental behaviour, altered enzymatic activity, lower growth rates, teratogenicity, carcinogenicity, and immunotoxicity[33, 37-39].

PCBs and OH-PCBs have been measured at detectable concentrations in a wide range of avian species[17, 87, 88]. When considering the wide range of toxic effects observed in wild birds, this evidence suggests a potential causal link between PCB and OH-PCB bioaccumulation and disruption of thyroid-dependent processes in wildlife populations[49], which could coincide with increased vulnerability of altricial nestlings to environmental contaminants[51]. PCBs and OH-PCBs have a high degree of structural similarity to triiodothyronine (T3) and thyroxine (T4) hormones[34, 89], which in birds are essential for controlling basal metabolic rate, pre-and postnatal differentiation of organ systems, regulation of body weight, muscle, and bone growth, initiation of moult and plumage growth, lipogenesis, and secondary sex characteristics[90]. European starlings (*Sturnus vulgaris*) and red-winged blackbirds (*Agelaius phoeniceus*), two altricial songbirds, are hatched without thermoregulatory responses, poor sensory capabilities, and have low levels of plasma T4 and T3. Critical development of thyroid function, coincident with the development of thermoregulatory capabilities, largely occurs during the period of nestling development. The risk of exposure and sensitivity to PCBs and OH-PCBs during the critical window of nestling development is likely greater than that of adults because young individuals undergo large and rapid structural and functional changes, making them particularly vulnerable to any adverse effects that may precipitate later in life[51, 52] .

We investigated how a developmental exposure to a commonly encountered EDC mixture, Aroclor 1254, may have effects on growth and physiological condition in nestling

European starlings and red-winged blackbirds. This study focused on the subtle effects following low dose exposure during critical early development on the endocrine system, namely through interactions with the hypothalamic-pituitary-thyroid axis. As part of a longer study following this cohort of birds through time, we hypothesize that PCBs would induce alterations in the HPT axis resulting in impaired thyroid function, which may be expressed as alterations in morphological and physiological responses during exposure, or that may only be realized in adulthood.

2.3 Methods

2.3.1 Study species

In 2011, 34 nestboxes (20.3 cm width, 15.2 cm depth, 70 cm high, entrance hole size 4.5 cm diameter) were established throughout the University of Saskatchewan's Goodale Research Farm (Saskatoon, Saskatchewan, Canada; 52°3' 23.13", -106° 30' 47.90"), where the land use is dominated by grazing pasture and limited cropland (wheat, canola, oats, and barley). The site attracts breeding European starlings (*S. vulgaris*), a widely distributed non-native passerine songbird used extensively in both laboratory and field-based research. Starlings readily adopt nestboxes and have a well understood breeding and migratory ecology[22, 75]. These characteristics, combined with their altricial development pattern (incubation time of 12 days, nestling period of 18 days[78]), make them highly suitable for examining morphological and physiological effects resulting from developmental exposure to PCBs[79]. The red-winged blackbird, also an altricial passerine (incubation time of 12-13 days, nestling period of 14 days[78]), is similarly suitable for studies of exposure to environmental contaminants. They are partial migrants[76], and construct semi-conspicuous nests at high densities in wetland areas[77], making it relatively easy to find a sufficient sample size of nests.

Starling nestboxes and blackbird open cup nests were monitored every 2-3 days for nesting activity and date of clutch initiation (appearance of first egg). The clutch was determined to be complete when a female bird was observed incubating the eggs or if the eggs were warm. At the end of the incubation period, nests were visited daily to determine precise hatching dates (day 0) and to initiate dosing (day 1). Dosing initiation was postponed by one day if hatching was asynchronous. Individual chicks within each nest were identified by clipping different downy feather tract patterns and were banded on day 7 for permanent identification.

2.3.2 Aroclor 1254 Dosing

Aroclor 1254 (Supelco Analytical, Bellefonte, PA; supplied by Sigma-Aldrich) was dissolved in food-grade organic sunflower oil (Compliments brand, Sobeys Canada) to produce three dosing solutions (50 ppm, 100 ppm, and 150 ppm) plus a sunflower oil only control (0 ppm) for the 2012 dosing study, while only two dosing solutions (50ppm, 100ppm) plus a sunflower oil control (0 ppm) were produced for the 2011 dosing study. Mean concentrations of Aroclor 1254 for all dosing solution batches were confirmed by chemical analysis (see supporting information for more detail, Table S2.1-S2.2). In 2011 and 2012, solutions were made from stock solutions of 1000 ppm (measured as 823.88 ± 30.41 µg/ml and as 987.47 ± 1.85 µg/ml, respectively) and serially diluted.

Dose volumes were adjusted and administered daily according to individual nestling body mass to maintain the following dosage levels based on nominal targets: 0 (control), 0.35 (low), and 0.70 (intermediate) µg Aroclor 1254/g-body weight [bw]/day [d], with a 1.05 (high) µg Aroclor 1254/g-body weight [bw]/day [d] dose being added for the second year (2012) of the study. The dosage levels chosen for both studies are environmentally relevant when compared to areas with

low Aroclor 1254 contamination and have been shown to produce measurable effects in other avian species[22, 91]. Nestlings were returned to the nests immediately after handling and dosing.

In 2011, a total of 21 starlings and 22 red-winged blackbirds were orally dosed daily from 1 to 18 and 1 to 14 days post-hatch, respectively, using a crop gavage needle. Nestling starlings (n = 7 control, 7 low, 7 intermediate) and nestling red-winged blackbirds (RWBL) (n = 6 control, n = 7 low, n = 9 intermediate) were randomly assigned to the different treatment groups within each nest to account for any nest or heritable effects. In 2012, eighty-four starlings only were orally dosed daily from 1 to 18 days post-hatch using a crop gavage needle. Nestling starlings (n = 21 birds/treatment) were randomly assigned to the four treatment groups within each box to account for any nest or heritable effects.

2.3.3 Morphological measurements and tissue collection

Starling body mass (± 0.1 g), bill length (mm), right tarsus length (mm), and right wing chord length (mm) were measured at hatch (day 1) and then at 4, 7, 10, 14, and 18 days post-hatch. Red-winged blackbird body mass (± 0.1 g), bill length (mm), right tarsus length (mm), and right wing chord length (mm) were measured at hatch (day 1) and then at 4, 7, 10, 14 days post-hatch. Differences in duration of dosing and measurements reflects the different patterns of ontogeny in the 2 species (nestling period is approximately 10-12 days in red-winged blackbirds, but 20-25 days in starlings). All data collection and recording was conducted by the same researcher to ensure consistency and was blind to experimental treatment. In 2012, body mass (± 0.1 g), bill length (mm), right and left tarsus length (mm), and right and left wing chord length (mm) were measured at hatch (day 1) and then at 4, 7, 10, 14, and 18 days post-hatch. All data collection and recording was conducted by the same researcher to ensure consistency and was

blind to experimental treatment. Fluctuating asymmetry of wing chord length (mm) and tarsus length (mm) were calculated as the absolute differences between right and left wing chord length, and right and left tarsus length, respectively.

When starlings and red-winged blackbirds reached 18 and 8 days post-hatch, respectively, they were transported to the Animal Care Unit (ACU) of the Western College of Veterinary Medicine (WCVM), University of Saskatchewan, Saskatoon, SK. Birds were held in cages with one nest per cage, in a room maintained under a summer photoperiod of 15L:09D (light:dark). On days 15 and 19, respectively, approximately 24h post dosing, a subset of red-winged blackbirds (n = 3 control, 3 low, 4 intermediate) and starlings (n = 2 control, 3 low, 3 intermediate) in 2011 were euthanized by CO_2 asphyxiation, and in 2012, a subset of starlings (n = 5 control, 5 low, 5 intermediate, 5 high) were similarly euthanized by CO_2 asphyxiation and livers were excised and stored frozen at -80°C for future analyses. In 2012, fifty-five birds remained in captivity for a future study examining migratory activity and orientation (Chapter 3).

2.3.4 Hormone analysis

Blood was sampled (maximum 0.3 ml) from starlings and red-winged blackbirds at 7 and 14 days-post-hatch in 2011 and 2012, and then from the subsets of euthanized birds, after birds were taken into captivity. Blood was collected by venipuncture of the jugular vein using a heparinized 26G needle and was transferred to microcentrifuge tubes. If sufficient blood volume could not be collected, the brachial vein was punctured and blood was collected into heparinised capillary tubes. Blood was kept on ice and centrifuged for 5 min (G-force = 3100) within 3 hours of collection to separate plasma. Samples were stored at -80°C until analysis. Total thyroxine (T4) and total triiodothyronine (T3) concentrations in 2012 starling plasma samples were

determined using enzyme-linked immunosorbent assay (ELISA) kits (Monobind 225-300 and 125-300, Lake Forest, CA 92630). Red-winged blackbird plasma was collected but not analyzed due to limitations of the assay and a decision to not pursue long-term studies on this species. Chicken plasma from an in-house 10 hen plasma pool was included in each plate and all samples were run in duplicate to measure inter- and intra-assay precision and reproducibility, respectively. The T3 assay had an average inter-assay coefficient of variation (CV) of 4.49% (n=5 assay plates) and the intra-assay coefficient of variation (CV) was 7.64% (n=2 replicates), however T4 samples were all below the assay detection limit (1.28 ng/ml).

2.3.5 Data analysis and statistics

Statistical analyses were performed using R version 3.0.0 (R Core Team 2014). Data was analyzed by one-sample Shapiro-Wilk test of normality. Aroclor 1254 treatment solutions and liver residues for both species and years were analyzed using a one-way analysis of variance (ANOVA), followed by a Tukey's post-hoc test to examine differences between treatments. Tests were deemed significant at $\alpha < 0.05$. Nestling survival (%) was analyzed using a Chi-square test to identify significant differences ($p < 0.05$) between treatment groups.

General linear mixed models were used to investigate the effects of Aroclor 1254 treatment on European starling (*S. vulgaris*) nestling growth from 1 to 18 days post-hatch and on red-winged blackbird nestling growth from 1 to 15 days post-hatch. Body mass, bill length, right wing chord length, right tarsus length, wing chord fluctuating asymmetry, tarsus length fluctuating asymmetry, and plasma total T3 concentrations were log- or square root-transformed to meet assumptions of normally-distributed residuals and analyzed using Gaussian distributions with the "nlme" package in R[92]. To improve model fit, quadratic (dph^2) and cubic (dph^3) time

components were added to the model where responses exhibited curvilinear trends. Treatment (i.e., Aroclor 1254 dose) and time were fixed effects; nest, subject, and the response slope over time for each subject were random effects which accounted for repeated measurements of individuals. We used Akaike's information criteria (AIC) to identify the best transformations and to decide whether to retain slope and intercept random effects[93]. Significant interactions were examined using post-hoc testing of contrasts in the "phia" package in R[94]. Data shown in figures represent untransformed means ± standard error of the mean (s.e.m.), and tests were deemed significant at $\alpha < 0.05$.

2.4 Results

2.4.1 Measured Aroclor 1254 in liver samples

Aroclor 1254 concentrations in liver tissues of 19 day old starlings and 15 day old red-winged blackbirds from 2011 did not differ significantly within treatment group due to very large inter-individual variation in liver concentrations among individuals from the same dose group (Table 2.1). Aroclor 1254 concentrations in liver tissues of 19 day old starlings from 2012 exhibited a dose dependent increase except for the low and intermediate dose groups which were equivalent ($p > 0.05$). Low, intermediate, and high dosed birds had liver residues significantly higher than controls ($p < 0.001$) but there was very large inter-individual variation in liver concentrations and no statistically significant differences in liver concentrations existed between any of the other dose groups.

Table 2.1. Summary of geometric means of nominal and measured concentrations of Aroclor 1254 in dosing solutions (µg/ml (ppm)) and livers (µg /g tissue (ppm) of nestling European starlings (EUST) and red-winged blackbirds (RWBL) by year. Liver residues were measured in euthanized day 19 starling and day 15 red-winged blackbird nestlings. Data reported as geometric means (min, max).

year	treatment	µg/g (ppm) b.w. oral dose	solution concentration (µg/ml (ppm))	EUST liver concentration (µg /g tissue (ppm))	*n*	RWBL liver concentration (µg /g tissue (ppm))	*n*
2011	control	0	1.87 ± 0.05	1.50 (0.67, 4.59)	3	2.02 (0.80, 5.12)	2
	low	50	21.61 ± 0.67	8.01 (3.16, 16.95)	3	10.04 (9.00, 11.19)	4
	intermediate	100	129.6 ± 4.45	12.68 (4.84, 26.75)	3	8.36 (4.57, 17.42)	4
	high	-	-	-	-	-	-
2012	control	0	0.03 ± 0.00	0.69 (0.22, 1.67)	5	-	-
	low	50	51.72 ± 0.02	13.12 (4.06, 59.00)	5	-	-
	intermediate	100	85.04 ± 0.02	13.25 (7.23, 32.43)	5	-	-
	high	150	153.1 ± 0.44	38.61 (17.67, 176.19)	4	-	-

2.4.2 European starling nestling survival, morphological and hormone measurements

In 2011, there were no effects of oral dosing on survivability of nestling starlings neither in the field (0 deaths) nor in captivity (χ^2 = 2.62, df = 2, p = 0.33), with no observed signs of toxicity. All birds, regardless of dose group, increased in size through time (Figures 2.1 and 2.2, Tables 2.2 and 2.3). Sex was not an important predictor and so was removed from statistical models. Thus, the most parsimonious models for body mass, right wing chord, and tarsus length indicated time (dph) to be an appropriate predictor. There was a significant increase in the body mass of all birds from 1 to 18 days post-hatch (β ± S.E.: 0.46 ± 0.0099, p < 0.001; Figure 2.1*a*, Table S2.3). There was no effect of Aroclor 1254 concentration on body condition in starlings pooled between years ($F_{1,26}$ = 0.36, p > 0.05); however, body condition was higher in starlings from 2012. Bill lengths increased over the treatment period between (β ± S.E.: 0.091 ± 0.011, p < 0.001; Figure 2.1*b*, Table S2.4) of all birds over the post-hatch period (1 – 18 dph). Wing chord length showed a linear increase over the treatment period from 1 to 18 days post-hatch (β ± S.E.: 0.39 ± 0.0083, p < 0.001; Figure 2.1*c*; Table S2.5). Tarsus lengths also showed a curvilinear increase over time from 1 to 18 days post-hatch (β ± S.E.: 0.27 ± 0.0069, p < 0.001; Figure 2.1*d*; Table S2.6).

The 2012 starlings from control and low treatment groups had 5% mortality (n = 1 dead/n = 22 total), while birds from the intermediate and high treatment groups had 12% mortality (n = 3 dead/n = 24 total) (Table 2.3). Overall, there were no significant effects of oral dosing on survivability (χ^2 = 1.83, df = 3, p = 0.61) in nestling starlings and no signs of toxicity observed in any of the birds. The most parsimonious models for body mass (Table S2.7), bill length (Table S2.8), right wing chord (Table S2.9), tarsus length (Table S2.10), and wing chord FA (Table S2.11), indicated time (dph) to be an important predictor, with both treatment and time

influencing tarsus length FA (Table S2.12). As above, sex was not included in statistical models. There was a significant and curvilinear increase in the mean body mass of all birds from 1 to 18 days post-hatch (β ± S.E.: 0.55 ± 0.011, $p < 0.001$; Figure 2.2*a*, Table S2.7). Bill lengths showed an increase over the treatment period between (β ± S.E.: 0.98 ± 0.015, $p < 0.001$; Figure 2.2*b*, Table S2.8) of all birds over the post-hatch period (1 - 18 dph). Wing chord length showed a linear increase over the treatment period from 1 to 18 days post-hatch (β ± S.E.: 0.15 ± 0.0015, $p < 0.001$; Figure 2.2*c*; Table S2.9). Tarsus lengths also showed a curvilinear increase over time from 1 to 18 days post-hatch (β ± S.E.: 0.21 ± 0.0022, $p < 0.001$; Figure 2.2*d*; Table S2.10). There were no significant effects of treatment on body mass, bill length, or right tarsus length; however, there was a trend towards an increase in wing chord length in the birds exposed to the 150 ppm treatment compared to the control group (β ± S.E.: 0.044 ± 0.025, $p = 0.08$; Figure 2.2*c*, Table S2.11).

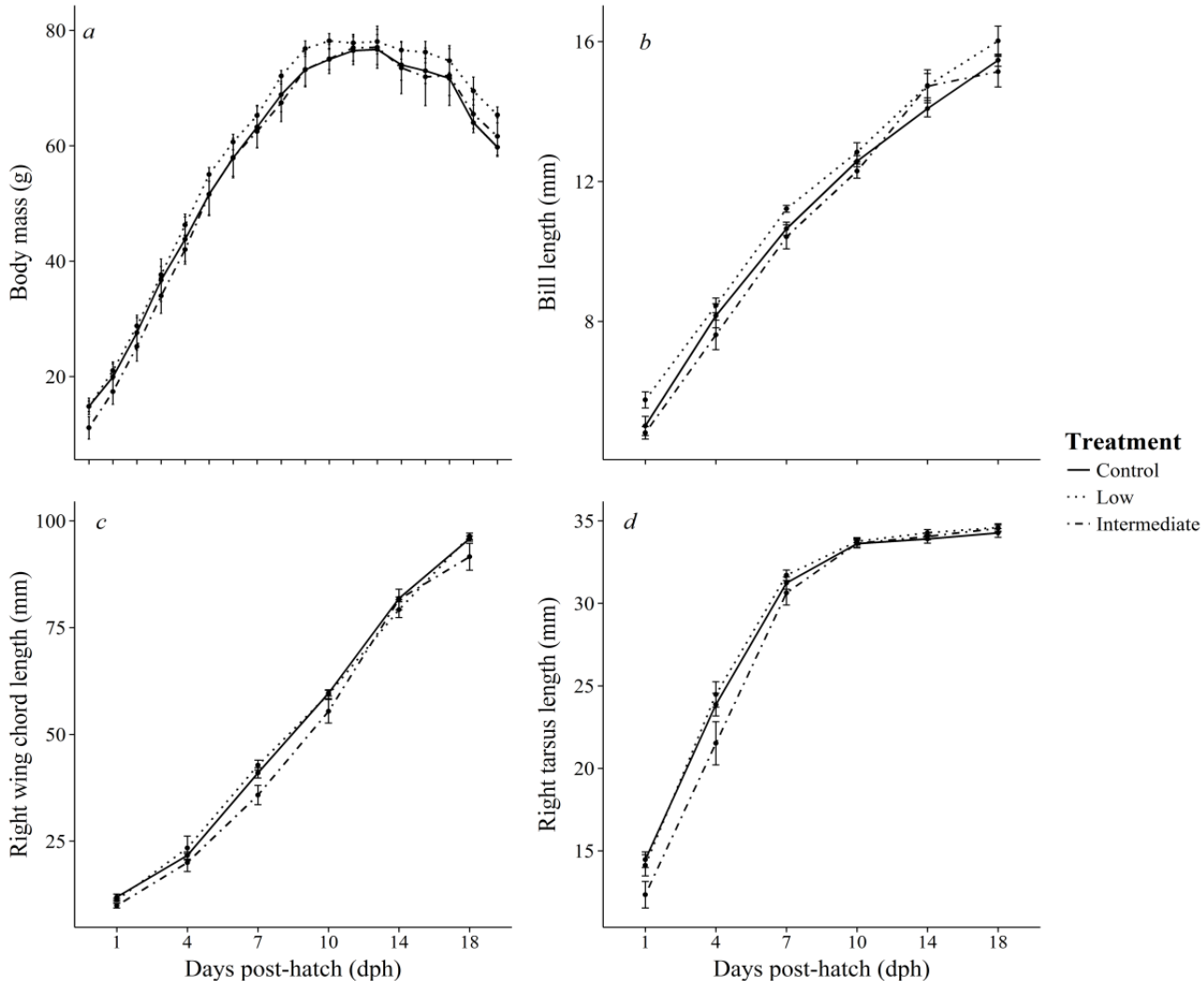

Figure 2.1. Body mass (g) (*a*), bill length (mm) (*b*), right wing chord length (mm) (*c*), and right tarsus length (mm) (*d*) measured from 1 to 18 days post-hatch in 2011 nestling European starlings exposed to 0 (control), 0.35 (low), or 0.70 (intermediate) µg Aroclor 1254/g-bw/day. Data reported as mean ± s.e.m.

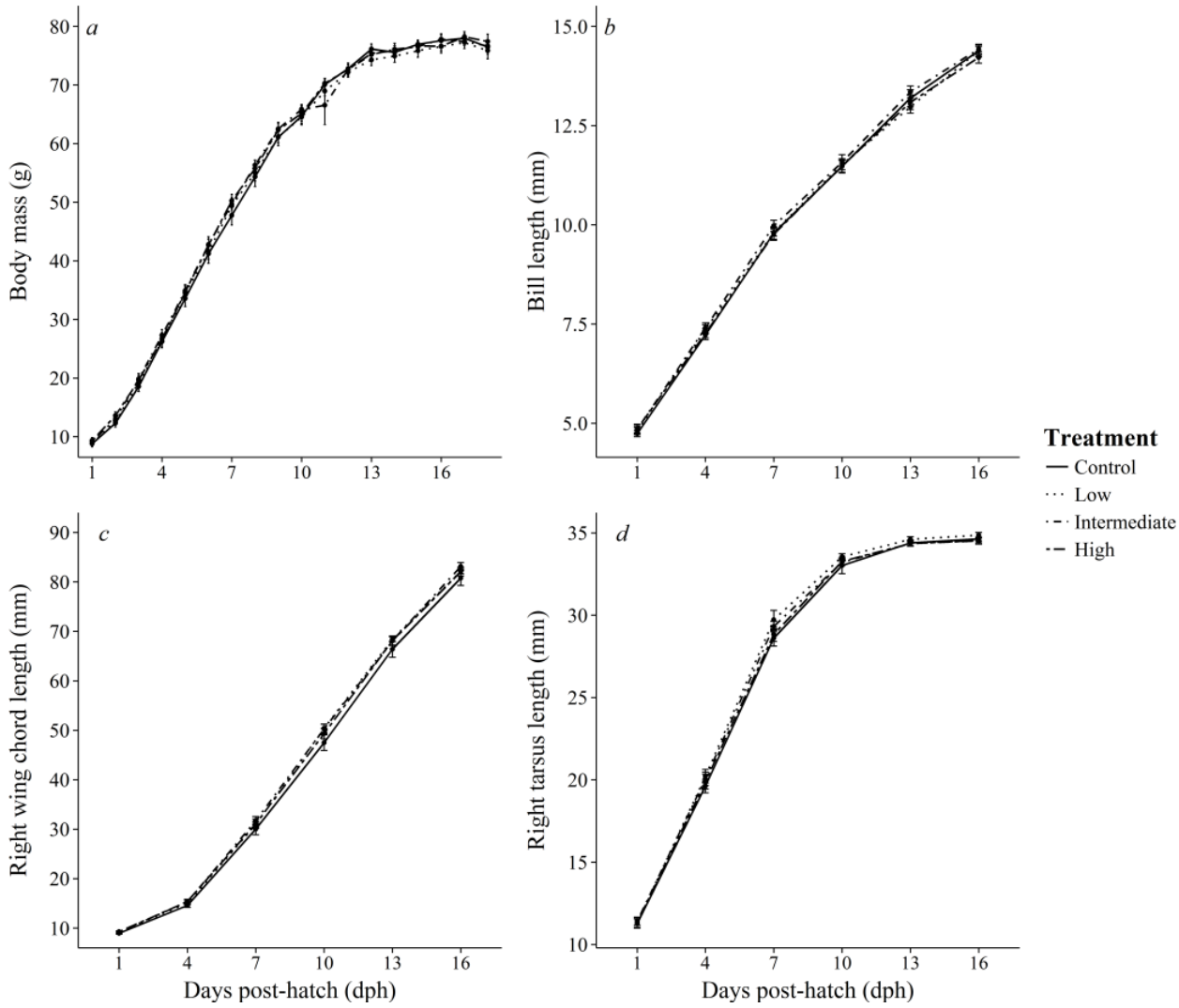

Figure 2.2. Body mass (g) (*a*), bill length (mm) (*b*), right wing chord length (mm) (*c*), and right tarsus length (mm) (*d*) measured from 1 to 18 days post-hatch in 2012 nestling European starlings exposed to 0 (control), 0.35 (low), 0.70 (intermediate), or 1.05 µg Aroclor 1254/g-bw/day. Data reported as mean ± s.e.m. There were no significant differences in right tarsus length, right wing chord length, bill length, or body mass among treatment groups (lines all overlapping).

Table 2.2. Mean morphological variables (mm) in 2011 nestling European starlings exposed to 0 (control), 0.35 (low), or 0.70 (intermediate) µg Aroclor 1254/g-bw/day at 18 days post-hatch. *n* = 7/treatment group. Data reported as mean ± s.e.m.

treatment	control	low	intermediate
body mass (g)	59.74 ± 1.60	65.34 ± 1.39	61.63 ± 3.25
bill length (mm)	15.47 ± 0.17	16.02 ± 0.42	15.14 ± 0.44
wing chord length (mm)	95.83 ± 0.60	96.4 ± 0.75	91.6 ± 3.16
tarsus length (mm)	34.27 ± 0.27	34.6 ± 0.23	34.54 ± 0.21
survival rate (%)	100	100	100

Table 2.3. Mean morphological variables (mm) in 2012 nestling European starlings exposed to 0 (control), 0.35 (low), 0.70 (intermediate), or 1.05 µg Aroclor 1254/g-bw/day at 18 days post-hatch. *n* = 21/treatment group. Data reported as mean ± s.e.m. * indicates significance when compared to control (0 ppm) birds; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.0001$.

treatment	control	low	intermediate	high
body mass (g)	77.56 ± 1.03	76.57 ± 1.12	77.80 ± 0.95	76.63 ± 0.79
bill length (mm)	14.39 ± 0.15	14.22 ± 0.14	14.39 ± 0.11	14.43 ± 0.12
wing chord length (mm)	80.79 ± 1.49	82.10 ± 0.88	82.14 ± 0.51	83.14 ± 0.79
tarsus length (mm)	34.63 ± 0.11	34.51 ± 0.20	34.86 ± 0.17	34.55 ± 0.15
wing chord asymmetry (mm)	0.74 ± 0.24	1.40 ± 0.29	1.57 ± 0.25	1.67 ± 0.34
tarsus length asymmetry (mm)	0.14 ± 0.03	0.33 ± 0.07	0.30 ± 0.05	0.36 ± 0.05
survival rate (%)	0.95	0.95	87.5	87.5

A two-way significant interaction existed between all treatments and time for wing chord fluctuating asymmetry (FA) ($p < 0.01$; Table S2.11), indicating that the slope of each treatment group was significantly different from the slope of the control group (Figure 2.3*a*). Fluctuating asymmetry of the wing chord significantly increased over time in birds from all treatment groups except controls ($p < 0.01$; Figure 2.3*a*, Table S2.11). Wing chord FA for birds from the low treatment group significantly increased over time ($z = 4.64$, $df = 1$, $p < 0.01$). Wing chord FA of birds from the intermediate treatment group significantly increased over time ($z = 5.58$, $df = 1$, $p < 0.01$). Wing chord FA of birds from the high treatment group significantly increased over time ($z = 5.23$, $df = 1$, $p < 0.01$). At day 10, birds from the intermediate and high treatment groups had differences in wing chord FA compared to control birds ($z = 4.03$, $df = 1$, $p = 0.01$) ($z = 5.11$, $df = 1$, $p < 0.01$), respectively. By day 13, birds from the low, intermediate, and high treatment groups all had larger wing chord FA relative to control birds ($z = 3.76$, $p < 0.05$), ($z = 3.85$, $df = 1$, $p < 0.05$), ($z = 4.21$, $df = 1$, $p < 0.01$). However, at day 16, these differences in asymmetry were no longer significant between treatment groups.

FA of the tarsus length showed a variable response over time, with no interactions between treatment and time (Figure 2.3*b*, Table S2.12). There were significant differences overall in tarsus FA in birds from the low and high treatment groups ($p < 0.01$, 0.05, respectively; Figure 2.3*b*, Table S2.12), indicating that birds from these treatment group had significantly larger differences in right and left tarsus measurements compared to controls.

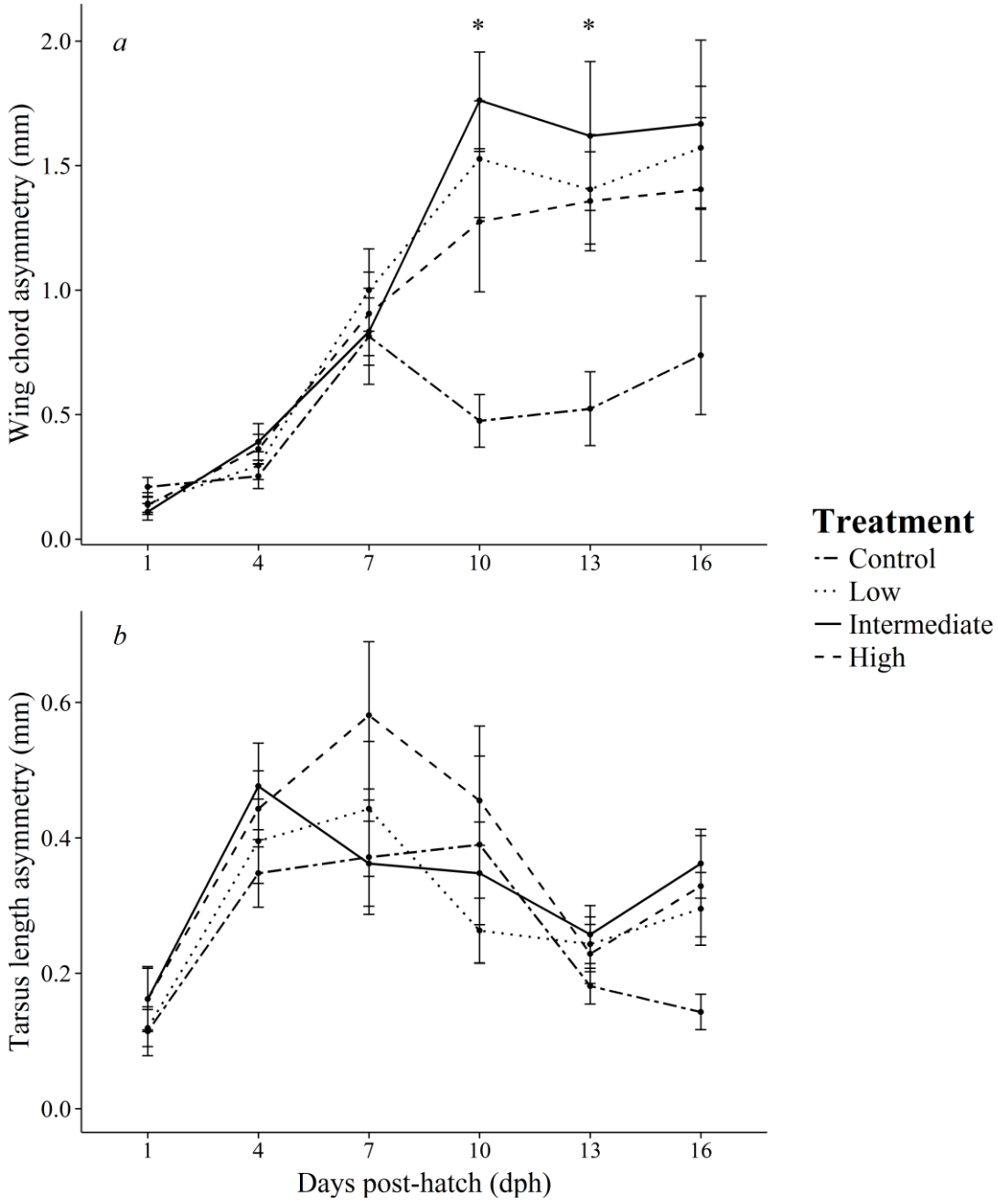

Figure 2.3. Fluctuating asymmetry (FA, mm) of wing chord length (*a*) and tarsus length (*b*) measured from 1 to 18 days post-hatch in 2012 nestling European starlings exposed to 0 (control), 0.35 (low), 0.70 (intermediate), or 1.05 µg Aroclor 1254/g-bw/day. FA is calculated as the absolute difference between length of left and right wing chord and tarsi, respectively. Data represented as mean ± s.e.m. Significant differences in wing chord FA between all treatment groups compared to control are indicated as: * $p < 0.05$.

Day 19 plasma hormone concentrations may reflect depressed levels from 2012 starlings given they were collected one day after being taken into captivity. Plasma total T3 (ng/ml) of all birds showed a significant increase between 7 and 14 days post-hatch (time: β ± S.E.: 0.18 ± 0.03, $p < 0.001$; Figure 2.4, Table S2.13), followed by a significant decrease between 14 and 19 days post-hatch (time: β ± S.E.: -0.008 ± 0.0014, $p < 0.001$; Figure 2.4, Table S2.13). Plasma total T4 concentrations were all below the detection levels of the assay (<1.28 ng/ml).

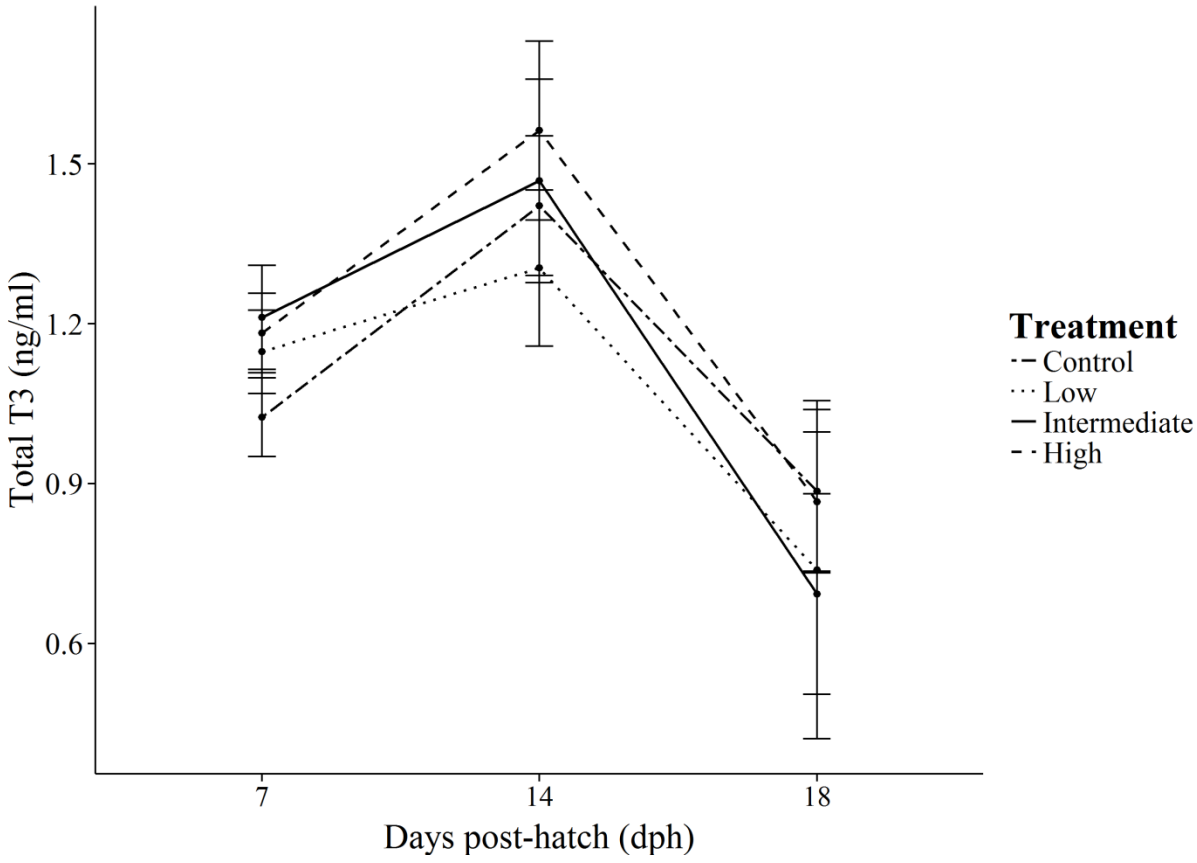

Figure 2.4. Plasma total triiodothyronine (T3) (ng/ml) measured from 1 to 18 days post-hatch in 2012 nestling European starlings exposed to 0 (control), 0.35 (low), 0.70 (intermediate), or 1.05 (high) µg Aroclor 1254/g-bw/day. Data reported as mean ± s.e.m. There were no significant differences T3 levels among treatment groups.

2.4.3 Red-winged blackbird nestling survival and morphological measurements

Morphological parameters for red-winged blackbirds at 15 days post-hatch are summarized in Table 2.4. Nestling red-winged blackbirds from 2011 had higher mortality overall, but this was not affected by dose group in the field (χ^2 = 0.13, df = 2, p = 0.94) nor in captivity (χ^2 = 1.79, df = 2, p = 0.47).

Table 2.4. Mean morphological variables (mm) in 2011 nestling red-winged blackbirds exposed to 0 (control), 0.35 (low), or 0.70 (intermediate) µg Aroclor 1254/g-bw/day at 14 days post-hatch. n = 6 control, 7 low, 9 intermediate. Data reported as mean ± s.e.m.

treatment	control	low	intermediate
body mass (g)	36.37 ± 3.50	42.44 ± 3.70	40.53 ± 1.85
bill length (mm)	10.05 ± 0.19	10.37 ± 0.35	9.97 ± 0.36
wing chord length (mm)	76.33 ± 1.09	76.11 ± 2.23	76.71 ± 2.78
tarsus length (mm)	32.27 ± 1.49	31.87 ± 0.75	32.37 ± 0.84
survival rate (%)	0.5	0.56	0.58

The most parsimonious models for body mass, right wing chord, and tarsus length indicated time (dph) and sex to be important predictors, with only time (dph) being a predictor for bill length. There was a significant increase in the body mass of all birds from 1 to 15 days post-hatch (β ± S.E.: 0.63 ± 0.015, p < 0.001; Figure 2.5*a*, Table S2.14). Body condition in red-winged blackbirds was not shown to be influenced by Aroclor 1254 concentrations in liver tissues ($F_{1,6}$ = 1.33, p > 0.05). Bill lengths increased over the treatment period (β ± S.E.: 0.45 ± 0.016, p < 0.001; Figure 2.5*b*, Table S2.15) of all birds over the post-hatch period (1 - 15 dph). Wing chord length showed a linear increase over the treatment period from 1 to 15 days post-hatch (β ± S.E.: 0.45 ± 0.0016, p < 0.001; Figure 2.5*c*; Table S2.16). Tarsus lengths also showed a curvilinear increase over time from 1 to 15 days post-hatch (β ± S.E.: 0.21 ± 0.0089, p < 0.001;

Figure 2.5*d*; Table S2.17). There were no significant effects of treatment on any of the morphological parameters; however, consistent with the dimorphism in the species, sex significantly predicted mass (β ± S.E.: 0.16 ± 0.0066, $p < 0.038$; Table S2.14) and right wing chord length (β ± S.E.: 5.11 ± 2.03, $p < 0.03$; Table S2.16). A two-way significant interaction existed between time and sex for right tarsus length ($p = 0.02$; Table S2.17), indicating that male tarsus length responded differently over time than females.

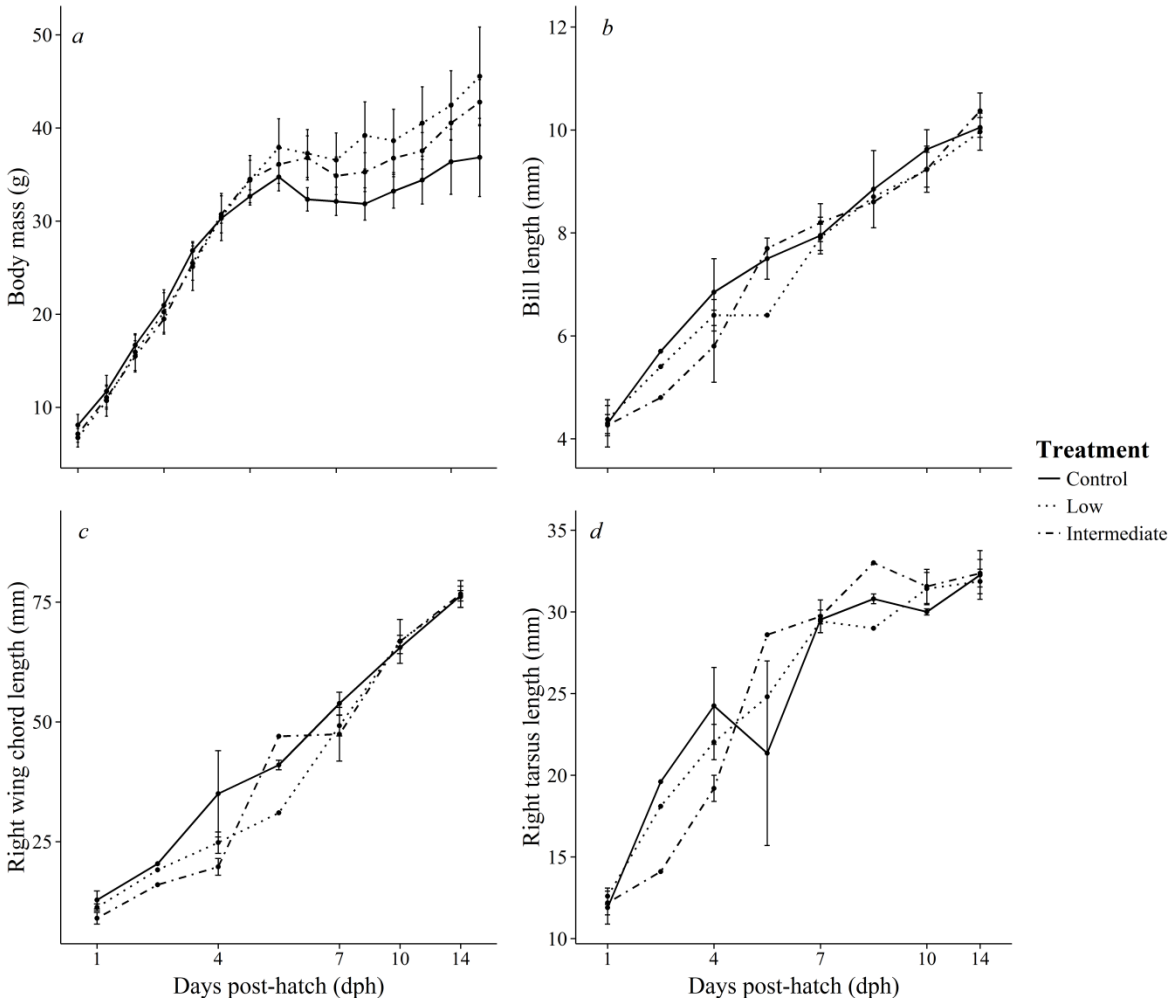

Figure 2.5. Body mass (g) (*a*), bill length (mm) (*b*), right wing chord length (mm) (*c*), and right tarsus length (mm) (*d*) measured from 1 to 15 days post-hatch in 2011 nestling red-winged blackbirds exposed to 0 (control), 0.35 (low), or 0.70 (intermediate) µg Aroclor 1254/g-bw/day. Data reported as mean ± s.e.m.

2.5 Discussion

2.5.1 Development of a passerine model for long-term studies

Preliminary data from our 2011 pilot study indicated that exposure to low levels of PCBs did not significantly affect survival or morphological parameters measured in both nestling European starlings and red-winged blackbirds over the post-hatch period. However, birds of both species were later tested in experimental trials on migratory behaviour which produced data that was inconclusive. Moreover, red-winged blackbirds proved unsuitable for long term studies of migratory orientation. These factors led to the implementation of a larger-scale study in 2012 using only European starlings, one more treatment level added, and a novel, more reliable method for assessing migratory activity and orientation trials (see Chapter 3).

Starlings exposed to ecologically relevant but low levels of PCBs showed significant increases in wing chord and tarsus length FA by days 10 and 13 post-hatch, while exhibiting no other adverse effects on other growth-related traits or plasma T3 concentrations. This suggested that early PCB exposure may have caused subtle impairment in physiological mechanisms that we did not detect, such as additional effects on the HPT axis or the hypothalamic-pituitary-adrenal (HPA) axis, both of which have been shown in other studies of birds[95, 96].

2.5.2 Impacts of PCB exposure on nestling development

Previous studies have reported a suite of developmental effects that can occur, such as embryonic mortality, teratogenicity, decreased hatching and fledgling success, decreased growth rates, and immunotoxicity[22, 39], all of which can have both immediate and long-lasting effects on chick viability, survival, and population-level success. No acute toxic effects or significant mortality resulting from early exposure to Aroclor 1254 was observed in either of our studies.

Similarly, Aroclor 1254 treatment did not alter any of the morphological traits measured except a trend towards increasing wing chord length in starlings from the high treatment group in 2012. These results are consistent with other avian studies, including an artificial exposure of nestling starlings exposed to a combination of EDCs including 17 β-estradiol, dibutyl phthalate, dioctyl phthalate, and bispenol A[33]. Although the authors measured reduced growth over the post-hatch period and a lower body mass in dosed birds at the end of the experiment, there were no effects on tarsus growth or wing length[33]. In another similar study, male starlings did exhibit compromised immune function and more complex song output, with female starlings preferring the song of males exposed to the mixture of EDCs. In addition, environmentally relevant exposures of Aroclor 1248 to adult female zebra finches (*Taeniopygia guttata*) have resulted in significant alterations in reproductive variables, such as an increased number of clutches laid and nests constructed by each breeding pair, incubation times per clutch, and increased mortality of nestlings hatched[97]. These alterations in reproductive variables indicate that PCBs may be directly interfering with hormonal regulation, at doses that do not produce acute signs of PCB toxicity, such as tremors, paralysis, nephromegaly, reduced heart, or spleen size.

2.5.3 Impacts of PCB exposure on plasma thyroid hormones

Exposure of experimental animals and wildlife to PCBs has resulted in a wide range of effects on thyroid function[34, 35], with consequences for thyroid gland morphology and function, thyroid hormone synthesis, secretion, metabolism, transport, binding, and excretion[30, 36]. Results of these studies have led to a rapid rise in wildlife studies reporting thyroid function as an indicator of environmental contamination from PCBs and similar compounds[34].

Plasma T3 concentrations in nestling starlings showed a similar response to other studies describing the response of thyroid hormones in starlings over the posthatch period, with the exception being that previous studies with nestling starlings exhibited peak T3/T4 levels between 10 and 12 days of age[48, 98] whereas we measured a sharp increase in T3 at 14 dph, which is concomitant with initial development of endothermy and resulting thermoregulatory control. T3 levels then showed a decrease to very low levels by the end of the experimental period at 19 dph, which is not supported by previous findings with nestling starlings. The sharp decrease in T3 levels may be attributed to the stress of transportation and subsequent introduction into captivity and so results need to be interpreted with caution. The lack of treatment-related effects on major morphological growth variables (body mass, bill length, wing chord, and tarsus length) supports our findings that T3 levels were not significantly altered by Aroclor 1254. Plasma T4 levels previously measured in nestling starlings are also initially very low, followed by a steady increase to concentrations found in adult birds[48]; however, we were unable to quantify plasma T4 concentrations due to the detection limit of the assay. Given the potential of PCBs and hydroxy-PCBs for thyroid disruption, future studies should aim to quantify thyroid gland T4 and use an assay with appropriate sensitivity for of quantification of total T4.

2.5.4 Fluctuating asymmetry

There has long been a need for a simple and reliable strategy to monitor stress in ecosystems affected by environmental pollutants[100]. One commonly used indicator of developmental instability, fluctuating asymmetry (FA), refers to small random deviations from the ideal bilateral symmetry of morphological traits[99]. Previous studies have reported an increase in FA in response to pollution-related stress across a wide variety of animals[100-102], suggesting its suitability as a sensitive biomarker of environmental stress[103]. Wing asymmetry in birds

adversely impacts aerodynamic performance, resulting in reductions in flight performance and increases the energetic costs of flight[104]. There were significant increases in wing chord fluctuating asymmetry in starlings exposed to low, medium, and high levels of Aroclor 1254 by 10 and 13 days post-hatch, but with no other overt effects on other morphological traits. Fluctuating asymmetry of wing chord and tarsus lengths did return to control levels by the end of the treatment period; however, further studies examining if asymmetry could persist and to what extent could this affect fitness and behaviour in juveniles are warranted. Our results indicate that while there could have been impacts on thyroid hormones that we were unable to measure, there also could have been deregulation of the hypothalamic-pituitary-adrenal axis (measured as corticosterone in birds), increasing the stress response and leading to wing asymmetry[101]. Previous studies exposing American kestrels to a dietary environmentally-relevant mixture of PCBs (Aroclors 1248: 1254: 1260; 1:1:1) found that these birds had significantly lower stress-induced corticosterone responses, along with significantly lower baseline levels of corticosterone[95]. This impairment of the corticosterone response system indicates that there may be a distinct disadvantage for wild birds which are chronically exposed to contaminants, as there may be negative carry-over effects in their ability to deal with environmental and physical stressors. While thyroid hormones are one of the main components of feather growth and moulting, impairment of the stress response in exposed starlings could further compound the relationship between PCB exposure and FA[102]. Increased asymmetry of tarsus length in birds exposed to low and high PCB treatments could also indicate impaired calcium metabolism. Exposure of rats and fish to Aroclor 1254 has been shown to significantly affect calcium metabolism, as demonstrated by alterations in normal bone development[105, 106]. Nestling pied flycatchers (*Ficedula hypoleuca*) and great tits (*Parus major*) from heavy metal polluted areas

have been shown to have increased tarsus and 3rd primary feather lengths, respectively[102]. In addition, blood concentration of organochlorine pollutants has been positively correlated with asymmetry of the third primary feathers in Arctic breeding glaucous gulls (*Larus hyperboreus*)[103]. This indicates that exposure to contaminants (heavy metals, hormone mimics) that have the ability to impair calcium metabolism could result in an increase in asymmetry of morphometrics in birds. Multiple studies of fluctuating asymmetry in birds exposed to contaminants, along with correlations throughout the literature between fluctuating asymmetry and genetic or environmental stressors[100], contribute to the suitability of our use of this measure to examine the effects of PCB-related stress on nestling starlings.

2.5.5 Large-scale relevance to avian populations

The results of this study highlight the need for further research examining how early exposures to endocrine disruptors during critical developmental windows could later affect key life stages in birds, such as migration. Much of the research examining the range of EDC effects on organisms has focused only on mechanistic toxicity pathways and consequent molecular, biochemical, and cellular responses during the exposure period[107], while failing to make extrapolations to adverse outcomes on whole individuals later in life and populations. This study focused solely on alterations in morphological and physiological responses to a developmental exposure to PCBs during the critical life stage of nestling development. While not conclusive here, any alterations in hormone homeostasis during development could result in long-term and irreversible changes in organ development, brain function, cognition, and behaviour[1]. Numerous ecotoxicology studies have made significant advances towards linking exposure to PCB mixtures with behavioural alterations in birds. Some of these include abnormal parenting behaviour such as inadequate incubation and nest defence[108], reduced nest attentiveness[109], altered courtship

behaviour[110], and decreased levels of brain dopamine and norepinephrine neurotransmitters[91]. Further examination of the relationship between PCB exposure and altered long-term developmental processes in birds is crucial for understanding the potential for latent effects on behaviours and life history events that are critical for survival and reproduction[111]. A subsequent study using the same European starlings from our 2012 cohort examined how this early exposure to Aroclor 1254 during nestling development could cause latent effects on the key life stage of migration (Flahr *et al*. 2014; Chapter 3). Juvenile birds (> 100 days post-hatch), when exposed to a 6-week photoperiod shift simulating an autumn migration, had a significant increase in mass, fat, moult score, and activity, indicating that migratory condition was successfully induced in captivity. However, high-dosed birds were significantly delayed in their ability to correctly orient when compared to the controls, and high-dosed males were significantly delayed in moult completion compared to untreated males. The findings from this follow-up study demonstrate that there is a link between early exposure to EDCs and latent alterations in migratory behaviour and those subtle effects from exposure did persist into adulthood. Impairment in behaviours critical for survival, such as breeding, reproduction, and migration, could lead to larger-scale negative consequences for populations in terms of health and survivability. The significance of these higher order effects is not well understood and is currently lacking in risk assessments of wildlife populations[1, 85, 112]. While we found that an artificial dosing study with PCBs does not necessarily result in overt acute effects on gross morphology, the alterations we observed in juvenile moult completion and migratory orientation in high-dosed birds followed later in life indicate that there are most likely subtle contaminant-induced alterations in hormone homeostasis during early development, which manifest as behavioural effects in exposed adults.

2.6 APPENDIX

Additional descriptions of chemical analyses and general linear mixed model (GLMM) statistical model output are provided.

Materials and Methods

Extraction and clean-up of dosing solutions

HPLC grade Acetone and Hexane, anhydrous sodium sulfate (certified ACS Granular) were purchased from Fisher Scientific (Fair Lawn, NJ, USA), Silica gel (pore size 60Å, 60-100 mesh, high-purity Davisil Grade 635) from Sigma-Aldrich (St. Louis, MO, USA). PCB standards were purchased from Wellington Laboratories, and included 27 mass labelled recovery and 5 mass labelled internal standards, a full list of native and mass labelled PCBs are given in the supporting materials (Table S2.1). Vials used in extraction were purchased from Chromspec (Chromatographic Specialties Inc., Brockville, ON, CA)

Dosing solution (100 µl) extractions were carried out following a method adapted from EPA Method 1668B (US-EPA 2008)[1]. All samples were fortified with 100ng/ml surrogate standard and extracted and cleaned using a multi-layer silica column (1g NaSO4, 2g basic silica (23% NaOH), 1g NaSO4, 4g acid Silica (30% sulphuric acid), 1g NaSO4), preconditioned with 150ml of *n*-Hexane. Samples were added to the column and were eluted with 250mL of *n*-hexane, collected in a round bottom flask, and rotary evaporated to 1ml. Extracts were transferred to 5ml vials with washings and concentrated under nitrogen to ~100 µl, and transferred with washings to GC vials and solvent exchanged to 10 µl of nonane with 100ng/ml labelled internal standard.

Identification and quantification of all target compounds was performed by GC/MS using an Agilent 7890A gas chromatograph equipped with an HT8 column (60 m x 0.25 mm i.d., 0.25 µm film thickness), connected to a 5975C mass spectrometer detector (MSD) operating using EI in SIM mode, (Agilent Technologies, Wilmington, DE, USA. Two µl were injected in splitless mode with an injector temperature of 250°C, and a helium flow of 1.5ml min^{-1}, GC oven temperature was initially 100°C held for 2 min, heated to 140°C at 20°C/min, to 200°C at 4°C/min, then to 300°C at 4°C/min and held for 17.5 min, the transfer line was maintained at 300°C. Aroclor concentrations were based on the concentrations of an Aroclor 1254 standard diluted in a 5-point calibration from 1 ng/ml to 2000 ng/ml. The R^2 of the native PCBs in the calibration curve was calculated to be > 0.99. Final concentrations for dosing solutions of Aroclor 1254 are expressed as µg Aroclor 1254/ml (ppm) and µg Aroclor 1254/g tissue (ppm).

Quality Assurance and Quality Control (QA/QC)

All equipment used was pre-cleaned with acetone and *n*-hexane to avoid sample contamination during cleanup, extraction, and chemical analysis, and where possible vials were baked at 450°C before use. A procedural blank was included for every 10 samples and was found to be 0 ng/g, indicating that there was no sample contamination.

Recovery concentrations ranged from 1.9% to 39.6% with a mean of 14.5%, the MDL was calculated as 3X the standard deviation plus the mean of blanks for each congener and in general the samples were above this indicating limited contamination during extraction.

Hexane blanks were included during each GC/MS run after every 6 samples to assess carryover and check the column condition. An internal standard was included after every 12 samples to

check retention times for shifts throughout the chromatogram and ensure correct PCB peak identification, any drift seen during a run and the samples were stopped.

References

1. US EPA 2008. 2008 Method 1668B: Chlorinated Biphenyl Congeners in Water, Soil, Sediment, Biosolids, and Tissue by HRGC/HRMS. Washington (DC): Engineering and Analysis Division, Office of Science and Technology, Office of Water, U.S. Environmental Protection Agency, 1-128.

Table S2.1. Native and mass labelled PCBs and the ions (m/z) used to monitor each.

	12C		13C	
Mono	188	190	200	202
Di	222	224	234	236
Tri	256	258	268	270
Tetra	290	292	302	304
Penta	324	326	337	339
Hexa	360	362	372	374
Hepta	394	396	406	408
Octa	428	430	440	442
Nona	462	464	474	476
Deca	496	500	508	510

Table S2.2. Nominal and measured concentrations (µg/ml) of Aroclor 1254 in each treatment and stock solution. Data represented as mean ± s.e.m. Significant differences between control and treatments are indicated as: * $p < 0.05$.

Nominal concentration µg/ml (ppm)	**Measured concentration (µg/ml (ppm))**
0 (control)	0.032 ± 0.00
50 (low)	51.72 ± 0.022
100 (intermediate)	85.04 ± 0.015
150 (high)	153.14 ± 0.441*
1000 (stock)	987.47 ± 1.85

Table S2.3: 2011 results of repeated measures generalized linear mixed model analyzing body mass in response to treatment and time. Body mass measured over 1 to 18 days post-hatch (dph) in 21 nestling European starlings exposed to 3 concentrations of Aroclor 1254/g-bw/day.

Fixed Effects	**β Estimate ± SE**	**t**	***p***	**df**
(Intercept)	2.10 ± 0.078	26.78	< 0.001	349
Treatment (Reference: 0 ppm)				
50ppm	0.046 ± 0.035	1.31	0.21	14
100ppm	0.010 ± 0.035	0.29	0.78	14
dph	0.46 ± 0.0099	46.62	< 0.001	349
dph^2	-0.030 ± 0.0011	-28.44	< 0.001	349
dph^3	0.00061 ±0.000037	16.69	< 0.001	349

Random Effects	**Variance**	**SD**
Nestbox (Intercept)	0.0052	0.072
BirdID (Intercept)	0.09	0.3
dph (Slope)	0.00035	0.019

Table S2.4: 2011 results of repeated measures generalized linear mixed model analyzing bill length in response to treatment and time. Bill length measured over 1 to 18 days post-hatch (dph) in 21 nestling European starlings exposed to 3 concentrations of Aroclor 1254/g-bw/day.

Fixed Effects	**β Estimate ± SE**	**t**	***p***	**df**
(Intercept)	2.36 ± 0.16	14.97	< 0.001	94
Treatment (Reference: 0 ppm)				
50ppm	0.11 ± 0.15	0.71	0.49	14
100ppm	0.20 ± 0.15	1.34	0.2	14
dph	0.091 ± 0.011	8.66	< 0.001	94

Random Effects	**Variance**	**SD**
Nestbox (Intercept)	0.028	0.17
BirdID (Intercept)	0.0012	0.035
dph (Slope)	-	-

Table S2.5: 2011 results of repeated measures generalized linear mixed model analyzing right wing chord length in response to treatment and time. Right wing chord length measured over 1 to 18 days post-hatch (dph) in 21 nestling European starlings exposed to 3 concentrations of Aroclor 1254/g-bw/day.

Fixed Effects	**β Estimate ± SE**	**t**	***p***	**df**
(Intercept)	3.15 ± 0.14	23.34	< 0.001	94
Treatment (Reference: 0 ppm)				
50ppm	0.069 ± 0.16	0.42	0.68	14
100ppm	-0.051 ± 0.16	-0.31	0.76	14
dph	0.39 ± 0.0083	47.73	< 0.001	94

Random Effects	**Variance**	**SD**
Nestbox (Intercept)	8.94×10^{-5}	0.0095
BirdID (Intercept)	4.58×10^{-2}	0.21
dph (Slope) -	-	

Table S2.6: 2011 results of repeated measures generalized linear mixed model analyzing right tarsus length in response to treatment and time. Tarsus length measured over 1 to 18 days post-hatch (dph) in 21 nestling European starlings exposed to 3 concentrations of Aroclor 1254/g-bw/day.

Fixed Effects	**β Estimate ± SE**	**t**	***p***	**df**
(Intercept)	2.32 ± 0.033	69.54	< 0.001	92
Treatment (Reference: 0 ppm)				
50ppm	0.021 ± 0.013	1.64	0.12	14
100ppm	0.012 ± 0.013	0.96	0.36	14
dph	0.27 ± 0.0069	39.27	< 0.001	92

Random Effects	**Variance**	**SD**
Nestbox (Intercept)	2.11 x 10-7	0.00046
BirdID (Intercept)	1.81 x 10-2	0.13
dph (Slope)	8.55 x 10-5	0.0092

Table S2.7: 2011 results of repeated measures generalized linear mixed model analyzing body mass in response to treatment and time. Body mass measured over 1 to 14 days post-hatch (dph) in 22 nestling red-winged blackbirds exposed to 3 concentrations of Aroclor 1254/g-bw/day.

Fixed Effects	**β Estimate ± SE**	**t**	***p***	**df**
(Intercept)	1.23 ± 0.093	13.27	< 0.001	300
Treatment (Reference: 0 ppm)				
50ppm	0.063 ± 0.076	0.84	0.42	9
100ppm	0.057 ± 0.072	0.8	0.45	9
dph	0.63 ± 0.015	40.94	< 0.001	300
dph^2	-0.059 ± 0.002	-29.99	< 0.001	300
dph^3	-0.0018 ± 0.00008	22.8	< 0.001	300
SexMale	0.16 ± 0.066	2.43	0.038	9

Random Effects	**Variance**	**SD**
Nestbox (Intercept)	0.01	0.1
BirdID (Intercept)	0.091	0.3
dph (Slope)	0.00097	0.031

Table S2.8: 2011 results of repeated measures generalized linear mixed model analyzing bill length in response to treatment and time. Body mass measured over 1 to 14 days post-hatch (dph) in 22 nestling red-winged blackbirds exposed to 3 concentrations of Aroclor 1254/g-bw/day.

Fixed Effects	**β Estimate ± SE**	**t**	***p***	**df**
(Intercept)	4.29 ± 0.31	13.81	< 0.001	79
Treatment (Reference: 0 ppm)				
50ppm	0.030 ± 0.33	0.091	0.93	9
100ppm	-0.14 ± 0.32	-0.44	0.67	9
dph	0.45 ± 0.016	27.54	< 0.001	79
SexMale	0.48 ± 0.29	1.66	0.13	9

Random Effects	**Variance**	**SD**
Nestbox (Intercept)	0.17	0.41
BirdID (Intercept)	0.17	0.42
dph (Slope)	-	-

Table S2.9: 2011 results of repeated measures generalized linear mixed model analyzing right wing chord length in response to treatment and time. Body mass measured over 1 to 14 days post-hatch (dph) in 22 nestling red-winged blackbirds exposed to 3 concentrations of Aroclor 1254/g-bw/day.

Fixed Effects	**β Estimate ± SE**	**t**	***p***	**df**
(Intercept)	9.50 ± 2.23	4.27	< 0.001	78
Treatment (Reference: 0 ppm)				
50ppm	-2.74 ± 2.41	-1.13	0.29	9
100ppm	-2.31 ± 2.29	-1.01	0.34	9
dph	0.45 ± 0.016	35.15	< 0.001	78
SexMale	5.11 ± 2.03	2.51	0.03	9

Random Effects	**Variance**	**SD**
Nestbox (Intercept)	2.95	1.72
BirdID (Intercept)	7.83	2.8
dph (Slope)	-	-

Table S2.10: 2011 results of repeated measures generalized linear mixed model analyzing right tarsus length in response to treatment and time. Body mass measured over 1 to 14 days post-hatch (dph) in 22 nestling red-winged blackbirds exposed to 3 concentrations of Aroclor 1254/g-bw/day.

Fixed Effects	**β Estimate ± SE**	**t**	***p***	**df**
(Intercept)	2.32 ± 0.042	54.88	< 0.001	77
Treatment (Reference: 0 ppm)				
50ppm	0.0098 ± 0.039	0.25	0.81	9
100ppm	0.014 ± 0.037	0.38	0.71	9
dph	0.21 ± 0.0089	23.56	< 0.001	77
dph^2	-0.0095 ± 0.00055	-17.1	< 0.001	77
SexMale	-0.055 ± 0.0089	-1.13	0.29	9
Sex x dph (Reference: Female 1dph)				
Male dph	0.011 ± 0.0048	2.39	0.02	77

Random Effects	**Variance**	**SD**
Nestbox (Intercept)	7.20×10^{-7}	0.00085
BirdID (Intercept)	2.53×10^{-3}	0.05
dph (Slope)	-	-

Table S2.11: 2012 results of repeated measures generalized linear mixed model analyzing body mass in response to treatment and time. Body mass measured over 1 to 18 days post-hatch (dph) in 84 nestling European starlings exposed to 4 concentrations of Aroclor 1254/g-bw/day.

Fixed Effects	**β Estimate ± SE**	**t**	***p***	**df**
(Intercept)	1.60 ± 0.036	44.99	< 0.001	932
Treatment (Reference: 0 ppm)				
50ppm	-0.013 ± 0.022	-0.58	0.57	37
100ppm	-0.020 ± 0.023	-0.87	0.39	37
150ppm	0.010 ± 0.022	0.46	0.65	37
dph	0.55 ± 0.011	50.41	< 0.001	932
dph^2	-0.037 ± 0.0013	-28.61	< 0.001	932
dph^3	0.00085 ± 0.000045	18.86	< 0.001	932

Random Effects	**Variance**	**SD**
Nestbox (Intercept)	3.08×10^{-4}	0.018
BirdID (Intercept)	0.025	0.16
dph (Slope)	1.25×10^{-4}	0.012

Table S2.12: 2012 results of repeated measures generalized linear mixed model analyzing bill length in response to treatment and time. Bill length measured over 1 to 18 days post-hatch (dph) in 84 nestling European starlings exposed to 4 concentrations of Aroclor 1254/g-bw/day.

Fixed Effects	**β Estimate ± SE**	**t**	***p***	**df**
(Intercept)	3.76 ± 0.11	34.26	< 0.001	414
Treatment (Reference: 0 ppm)				
50ppm	0.051 ± 0.12	0.42	0.67	62
100ppm	0.086 ± 0.12	0.71	0.48	62
150ppm	0.15 ± 0.12	1.26	0.21	62
dph	0.98 ± 0.015	66.22	< 0.001	414
dph^2	-0.020 ± 0.00082	-24.46	< 0.001	414

Random Effects	**Variance**	**SD**
Nestbox (Intercept)	0.058	0.24
BirdID (Intercept)	0.068	0.26
dph (Slope)	0.00091	0.030

Table S2.13: 2012 results of repeated measures generalized linear mixed model analyzing right wing chord length in response to treatment and time. Right wing chord length measured over 1 to 18 days post-hatch (dph) in 84 nestling European starlings exposed to 4 concentrations of Aroclor 1254/g-bw/day.

Fixed Effects	**β Estimate ± SE**	**t**	***p***	**df**
(Intercept)	2.15 ± 0.023	92.58	< 0.001	415
Treatment (Reference: 0 ppm)				
50ppm	0.035 ± 0.025	1.40	0.17	62
100ppm	0.038 ± 0.025	1.52	0.13	62
150ppm	0.044 ± 0.025	1.78	0.08	62
dph	0.15 ± 0.0015	98.08	< 0.001	415

Random Effects	**Variance**	**SD**
Nestbox (Intercept)	0.0010	0.032
BirdID (Intercept)	0.0011	0.033
dph (Slope)	-	-

Table S2.14: 2012 results of repeated measures generalized linear mixed model analyzing right tarsus length in response to treatment and time. Tarsus length measured over 1 to 18 days post-hatch (dph) in 84 nestling European starlings exposed to 4 concentrations of Aroclor 1254/g-bw/day.

Fixed Effects	**β Estimate ± SE**	**t**	***p***	**df**
(Intercept)	2.24 ± 0.015	152.11	< 0.001	414
Treatment (Reference: 0 ppm)				
50ppm	0.0021 ± 0.011	0.19	0.85	62
100ppm	0.014 ± 0.011	1.28	0.2	62
150ppm	0.0038 ± 0.011	0.34	0.73	62
dph	0.21 ± 0.0022	95.67	< 0.001	414
dph^2	-0.0083 ± 0.00012	-69.83	< 0.001	414

Random Effects	**Variance**	**SD**
Nestbox (Intercept)	1.05×10^{-11}	3.24×10^{-6}
BirdID (Intercept)	0.0097	0.098
dph (Slope)	4.43×10^{-5}	0.0067

Table S2.15: 2012 results of repeated measures generalized linear mixed model analyzing fluctuating asymmetry of wing chord in response to treatment and time. Wing chord asymmetry measured over 1 to 18 days post-hatch (dph) in 84 nestling European starlings exposed to 4 concentrations of Aroclor 1254/g-bw/day.

Fixed Effects	**β Estimate ± SE**	**t**	***p***	**df**
(Intercept)	0.44 ± 0.081	5.5	< 0.001	412
Treatment (Reference: 0 ppm)				
50ppm	-0.091 ± 0.11	-0.79	0.43	62
100ppm	-0.12 ± 0.11	-1.071	0.29	62
150ppm	-0.13 ± 0.11	-1.15	0.26	62
dph	0.038 ± 0.012	1.14	0.26	412
Treatment x dph (Reference: 0ppm 1dph)				
50ppm dph	0.038 ± 0.012	3.08	0.0022	412
100ppm dph	0.049 ± 0.012	3.91	0.0001	412
150ppm dph	0.052 ± 0.012	4.17	< 0.001	412

Random Effects	**Variance**	**SD**
Nestbox (Intercept)	6.78×10^{-5}	0.0082
BirdID (Intercept)	-	-
dph (Slope)	2.43×10^{-4}	0.016

Table S2.16: 2012 results of repeated measures generalized linear mixed model analyzing fluctuating asymmetry of tarsus length in response to treatment and time. Tarsus length asymmetry measured over 1 to 18 days post-hatch (dph) in 84 nestling European starlings exposed to 4 concentrations of Aroclor 1254/g-bw/day.

Fixed Effects	**β Estimate ± SE**	**t**	***p***	**df**
(Intercept)	0.061 ± 0.049	1.23	0.22	413
Treatment (Reference: 0 ppm)				
50ppm	0.10 ± 0.037	2.84	0.0061	62
100ppm	0.046 ± 0.037	1.25	0.22	62
150ppm	0.079 ± 0.037	2.15	0.035	62
dph	0.20 ± 0.024	8.19	< 0.001	413
dph^2	-0.024 ± 0.0034	-7.05	< 0.001	413
dph^3	0.00079 ± 0.00013	6.09	< 0.001	413

Random Effects	**Variance**	**SD**
Nestbox (Intercept)	4.84×10^{-6}	0.0022
BirdID (Intercept)	-	-
dph (Slope)	4.58×10^{-5}	0.26

Table S2.17: 2012 results of repeated measures generalized linear mixed model analyzing plasma total triiodothyronine (T3) (ng/ml) in response to treatment and time. Total mean T3 (ng/ml) measured over 1 to 18 days post-hatch (dph) in 84 nestling European starlings exposed to 4 concentrations of Aroclor 1254/g-bw/day.

Fixed Effects	**β Estimate ± SE**	**t**	***p***	**df**
(Intercept)	0.16 ± 0.17	0.92	0.36	75
Treatment (Reference: 0 ppm)				
50ppm	0.036 ± 0.046	0.77	0.45	40
100ppm	-0.020 ± 0.047	-0.42	0.68	40
150ppm	0.025 ± 0.049	0.5	0.62	40
dph	0.18 ± 0.033	5.57	< 0.001	75
dph^2	-0.0080 ± 0.0014	-5.58	< 0.001	75

Random Effects	**Variance**	**SD**
Nestbox (Intercept)	0.0085	0.092
BirdID (Intercept)	-	-
dph (Slope)	5.57×10^{-8}	0.00024

3 CHAPTER 3

Developmental exposure to Aroclor 1254 alters migratory behaviour in juvenile European starlings (*Sturnus vulgaris*)

Preface

This chapter was submitted to Environmental Science and Technology under joint authorship with Leanne M. Flahr (Toxicology Graduate Program, Toxicology Centre, University of Saskatchewan), Nicole L. Michel (School of Environment and Sustainability, University of Saskatchewan), Alexander R. D. Zahara (Department of Biology, University of Saskatchewan), Paul D. Jones (Toxicology Centre & School of Environment and Sustainability, University of Saskatchewan), and Christy A. Morrissey (Toxicology Centre & Department of Biology, University of Saskatchewan). The tables, figures, and references cited in this article have been re-formatted here to the thesis style. References cited in this chapter are listed in the reference section of this thesis.

Study contributions

The design of the 2011 and 2012 nestling dosing studies, conduction of field work, captive work, laboratory work, primary data analysis, and writing of manuscript was conducted by Leanne M. Flahr. Christy Morrissey, Principle Investigator, was also directly involved in conceiving and designing the studies, provided training of first author, funding, assistance both in the field and captivity, and editing of the manuscript. Nicole Michel provided guidance and assistance with the statistical models and data analysis. Alexander Zahara acted as a field assistant with the entire 2012 field and captive studies with input on the study design and interpretation. Garry

Codling provided assistance in GC/MS analysis of dosing solution and liver residue Aroclor 1254 concentrations, and with analysis of final data output. Paul Jones provided assistance with modification of method adapted from EPA Method 1668B (US-EPA 2008) for GC/MS analysis of dosing solution and liver residue Aroclor 1254 concentrations.

The study reported here was approved by the University of Saskatchewan's Animal Research Ethics Board and adheres to the Canadian Council on Animal Care guidelines for humane animal use and the University of Saskatchewan Policy on Care and Use of Animals in Research (Animal Use Protocol #20110043).

3.1 Abstract

Birds exposed to endocrine disrupting chemicals during development could be susceptible to neurological and other physiological changes affecting migratory behaviours. We investigated the effects of Aroclor 1254, a polychlorinated biphenyl (PCB) mixture, on moult, fattening, migratory activity and orientation in juvenile European starlings (*Sturnus vulgaris*). Birds were orally administered Aroclor 1254 from 1 through 18 days-post-hatch and later exposed in captivity to a photoperiod shift simulating an autumn migration. Migratory activity and orientation were examined using Emlen funnel trials. Across treatment groups, we found a significant increase in mass, fat, and moulting, and decreasing plasma thyroid hormones over time. We observed a significant increase in activity as photoperiod was shifted from 13L:11D (light:dark) to 12L:12D, demonstrating that migratory condition was induced in captivity. At 12L:12D, control birds showed a directional preference for 155.95° (South-southeast), while high-dosed birds did not. High-dosed birds showed a delayed directional preference for 197.48° (South-southwest) under 10L:14D, concomitant with apparent delays in moult. These findings link alterations in avian migratory behaviour to contaminant-specific mechanisms. Subtle contaminant-induced alterations in hormone homeostasis during early development could lead to larger-scale effects, including changes in migratory activity and orientation behaviour, which may be contributing to observed global declines in migratory species.

3.2 Introduction

Worldwide, many migratory bird species are exhibiting sustained and expansive population declines, prompting research into the likely causal factors influencing mortality during migration. The migratory period is often thought to carry higher mortality risk, which for

at least one passerine species was 15 times higher on migration than any other period in the annual life cycle[113]. Important avian life history events such as moult and migration are sensitive to seasonal environmental cues with a cascade of internal physiological changes that are under direct hormonal and neurological control. The rate of energy deposition, timing of departure, duration of migration, fuel consumption, orientation, and navigational abilities are all considered essential components of successful migration[114]. However, these factors are potentially vulnerable to subtle impairment of the morphological, physiological, and behavioural traits expressed before, during, and after migration[112].

Chemical pollutants produced by anthropogenic activity are widespread in the environment and are commonly found in the tissues of wildlife and humans[1, 2]. Endocrine disrupting chemicals (EDCs) are a group of highly heterogeneous exogenous compounds that are biologically active, capable of mimicking the action of endogenous hormones, and have the potential to interfere with homeostatic systems of organisms at multiple life stages including migration[3, 4]. Much of the research examining the range of EDC effects on organisms has focused on mechanistic toxicity pathways and consequent molecular, biochemical, and cellular responses [107], while failing to make extrapolations to adverse outcomes on whole individuals and populations. The significance of these higher order effects is not well understood and is currently lacking in risk assessments of avian wildlife populations[1, 85].

Polychlorinated biphenyls (PCBs) are ubiquitous synthetic organic contaminants[2] that were previously manufactured and marketed as commercial Aroclor mixtures[13]. PCBs are pervasive environmental contaminants[21, 22] due to their persistence and lipophilic properties that facilitate bioaccumulation and biomagnification through food chains[2]. In addition to PCBs, currently used industrial chemicals such as flame retardants, plasticizers, pesticides, and

pharmaceutical products, can also act as endocrine disruptors[3]. The structurally similar properties of several PCBs and their toxic hydroxylated metabolites (OH-PCBs) to thyroid hormones have led to wide ranging effects on processes common between humans and experimental animals, such as impairments in learning, cognitive abilities, memory, and behaviour. Their occurrence at detectable concentrations in a wide range of avian species[40] may contribute to disruption of thyroid-dependent processes in avian wildlife populations.

In birds, normal levels of thyroid hormones are essential for controlling basal metabolic rate, pre-and post-hatch differentiation of organ systems, growth, initiation of moult and plumage growth, lipogenesis, and secondary sex characteristics[45]. They are also important for nervous system and brain development[36, 47]. The effects of PCBs and other dioxin-like compounds on brain development (e.g. asymmetry) in birds have been characterized[19], which could have negative consequences for migratory ability later in life. We hypothesize that subtle alterations in physiological processes from EDC exposure during critical developmental windows may have consequences for successful moult and migration later in life, namely through interference with the hypothalamic-pituitary-thyroid (HPT) axis[70]. Alterations in the HPT axis can have consequences for impaired thyroid function, which may be expressed in two ways: 1) alterations in physiological and morphological responses during developmental exposure and 2) subsequent effects on behaviour as an adult. Our objective is to evaluate how early exposure to a commonly encountered PCB mixture, Aroclor 1254, may cause latent effects on a key life stage of migration. We used a controlled dosing and captive study to evaluate potential effects on moult, fattening, activity, and orientation behaviour in a migratory songbird model, the European starling (*Sturnus vulgaris*), under a simulated autumn migration.

3.3 Methods

3.3.1 Study animals and husbandry

In 2011, 34 nestboxes (20.3 cm width, 15.2 cm depth, 70 cm high, entrance hole size 4.5 cm diameter) were established throughout the University of Saskatchewan's Goodale Research Farm (Saskatoon, Saskatchewan, Canada; 52°3' 23.13", -106° 30' 47.90"). Breeding European starlings (*S. vulgaris*) readily adopt nestboxes and are diurnal migrants, with a well-studied breeding and migratory ecology[22, 75]. These characteristics, combined with their intelligence and robustness in captivity, make them highly suitable for examining changes in migratory behaviour resulting from exposure to PCBs[79].

Nestboxes were monitored every 2-3 days for nesting activity and date of clutch initiation (appearance of first egg). The clutch was determined to be complete when a female starling was observed incubating the eggs or if the eggs were warm. At the end of the 12-day incubation period, nestboxes (n = 15) were visited daily to determine precise hatching dates (day 0) and to initiate dosing (day 1). Individual chicks within each nest were identified by clipping different downy feather tract patterns and were banded on day 7. On day 18, nestling starlings were brought into captivity and caged as a nest group at the Animal Care Unit at the Western College of Veterinary Medicine, University of Saskatchewan. Nestlings were fed an organic wetted mixture of turkey starter crumbles, hard-boiled eggs, pureed carrots, and multivitamin powder (Hagen Prime) 6-8 times a day to satiation until they were able to independently feed on dry turkey starter (approx. age 30-35d). Colour-banded birds were then group housed in a free flight colony room (5 x 3 x 3 m) for 2 months before being randomly assigned to cages with 2-3 birds per cage. Starlings were maintained on *ad libitum* water and turkey starter crumbles

supplemented with fresh fruit and mealworms under a summer photoperiod of 15L:09D (light: dark) (lights on 06:00 hours) to mimic natural daylength until initiation of photoperiod shifts.

3.3.2 Aroclor 1254 dosing

Treatment levels were chosen to mimic an environmentally relevant exposure that would produce subtle effects in the study species, while not producing overt signs of PCB toxicity. Analytical grade Aroclor 1254 (Supelco Analytical, Bellefonte, PA) was dissolved in food-grade organic sunflower oil (Compliments brand, Sobeys Canada) to produce a sunflower oil only control and three dose levels (50 ppm, 100 ppm, and 150 ppm). Mean concentrations of Aroclor 1254 for all dosing solution batches were confirmed by chemical analysis (see supporting information and Table S3.1 for more detail) as 0 ppm (control) = 0.03 ± 0.00005 µg/ml, 50 ppm (low) = 51.72 ± 0.02 µg/ml, 100 ppm (intermediate) = 85.04 ± 0.02 µg/ml, and 150 ppm (high) = 153.1 ± 0.44 µg/ml. Solutions were made from a stock of 1000 ppm (= 987.47 ± 1.85 µg/ml) (Table S3.2).

Nestlings (n = 21 birds/treatment) were randomly assigned to the four treatment groups within each box to account for any nest or heritable effects. Eighty-four birds were orally dosed daily from 1 to 18 days post-hatch using a crop gavage needle. Dose volumes were adjusted daily according to nestling body mass to maintain the following dosage levels based on nominal targets: 0 (control), 0.35 (low), 0.70 (intermediate), and 1.05 (high) µg Aroclor 1254/g-body weight [bw]/day [d]. On day 19, approximately 24h post-dosing, a subset of starlings (n = 5) from each treatment group were euthanized by CO_2 asphyxiation. A total of 55 birds remained for Emlen funnel migration trials.

3.3.3 Emlen funnel migration trials

Plastic flower pots (diameter 38.1 cm, height 16.5 cm) were used to create a modification of the original Emlen funnel[67] to test the birds' migratory orientation. Emlen funnels are circular orientation cages with an ideal slope that ensures a bird is forced to return to the center after an activity bout, yet large enough so that movement from the center to the edge of the funnel is easily distinguished by observation with overhead cameras. The top opening is covered with a fine-meshed screen, allowing individual birds to see the sky, while a bottom opening is cut into the funnel for easy placement and removal of birds. Funnels were spray-painted white to obtain contrast between the bird and the funnel. A black marking was placed on the edge of each funnel (not visible to the bird), aligned to magnetic north. To track migratory movements, digital cameras (ADS-180, Swann Communications) were securely attached to the end of a 10ft aluminum pole overhanging the funnels. Each camera recorded the movements from six funnels simultaneously, and five cameras were connected to a single digital video recorder (DVR8-2550, Swann Communications) and a computer monitor.

Captive starlings were put on a regime of gradually decreasing photoperiod over six weeks from 15L:09D, with weekly 1-hr time shifts to 09L:15D. The time of light shifting once weekly matched the natural outdoor sunrise over the experimental period but shortened the photoperiod by advancing the daily sunset. The orientation experiments took place outdoors in a large open field with no landmarks visible from the funnels. Funnel tests occurred over two consecutive days each week during each photoperiod regime, with half of the 55 birds randomly tested on the first day and half tested on the second day (see Table S3.3 for breakdown of *n*/sex for each trial). In some cases, a bird was excluded from a trial if it had any sign of injury, typically from damage to flight feathers. Birds were allowed to feed in their home cages for 45

minutes after lights turned on and were then, at random, individually placed into inverted funnels and transported immediately to the experimental site without visual cues. Funnel trials occurred under clear skies and within four hours of the natural sunrise, as required for diurnally migratory starlings[59]. Trials lasted for 30 minutes, with the first five minutes omitted during analysis to allow acclimation to the funnels.

3.3.4 Migratory condition measurements

Body mass (± 0.1 g), furcular fat score[115], and primary moult score[116] were measured twice weekly from the start (15L:09D) to the end of the photoperiod shift (09L:15D). Data collection and recording was conducted by the same researcher to ensure consistency and was blind to experimental treatment. Measurements obtained from each bird took place on days that did not coincide with funnel trials and were averaged for each week. Furcular fat deposits were scored on a scale from 0 – 5 (0 = no fat deposit, 5 = convex bulge of fat in the furcular region). A primary feather moult score for each bird was calculated as the sum of growth scores over nine primary wing feathers. Each primary feather on the right wing was assigned a score from 0 – 5 (0 = old feather, 5 = fully grown new feather), for a maximum wing moult score of 45.

3.3.5 Hormone analysis

Blood was collected (maximum 0.3 ml) from each bird on weeks 1, 4, and 6 of the experimental period by venipuncture of the jugular vein using a 26G needle and was transferred to heparinized microcentrifuge tubes. Blood was kept on ice and centrifuged for 5 min (G-force = 3099) within 3 hours of collection to separate plasma. Samples were stored at -80°C until analysis. Total thyroxine (T4) and total triiodothyronine (T3) concentrations in plasma samples were determined using enzyme-linked immunosorbent assay (ELISA) kits (Monobind 225-300 and 125-300, Lake

Forest, CA 92630). Chicken plasma from an in-house 10 hen plasma pool was included in each plate and all samples were run in duplicate to measure inter- and intra-assay precision and reproducibility, respectively. The T3 assay had an average inter-assay coefficient of variation (CV) of 5.96% (n = 5 replicates) and the intra-assay CV was 8.81% (n = 2 replicates), however T4 samples were all below detection.

3.3.6 Data analysis and statistics

General linear mixed models (GLMM) were used to investigate the effects of Aroclor 1254 treatment on European starling measurements and activity over the course of the photoperiod shifts. Body mass, furcular fat score, right wing chord length, plasma total T3 concentrations, and migratory activity were log- or square root-transformed to meet assumptions of normally-distributed residuals and analyzed using Gaussian distributions with the "nlme" package[92] in R version 3.0.0 (R Core Team 2014). To improve model fit, quadratic ($Photoperiod^2$) and cubic ($Photoperiod^3$) time components were added to the model where responses exhibited curvilinear trends. Photoperiod, treatment, and sex were fixed effects; nestbox, subject, camera/funnel position, and the response slope over time for each subject were random effects. We used Akaike's information criterion (AIC) to identify the best transformations and to decide whether to retain slope and/or intercept random effects[93]. Significant interactions were examined using post-hoc testing of contrasts in the "phia" package[94]. Data shown in figures represents untransformed means ± standard errors of the mean (s.e.m.), and tests were deemed significant at $\alpha < 0.05$.

Emlen funnel video files were analyzed with BirdOriTrack software, a custom-designed motion video-tracking program[71] that allows for the simultaneous analysis of 6 birds (funnels)

and corrects for camera distortion. This program obtains the vector orientations and distance moved for each bird relative to the center of each funnel by comparing neighbouring video frames. Output from the program includes the unimodal and axial mean direction (θ), mean vector length (r), the number of counted movements (*n*), and the total activity of the bird for each funnel. The total number of counted movements (*n*) refers to the number of hops that the bird completes in the funnel during the trial period. The measure of total activity in the funnel takes into account both the presence of a valid hop (*n*) in the funnel and the mean vector length (r), which best represents the movements of migratory activity in the funnel. Activity is calculated as the length of each bird's movement over a set time interval (5 seconds) relative to the radius of the funnel (radius = 1). Output from BirdOriTrack was used for calculating circular statistics to determine the mean orientation of all birds from each trial and treatment group using Oriana 3.0 software (Kovach Computing Services). A Rao's spacing U test was first used to compare orientation distributions among treatment groups and trials to determine whether mean orientation differed significantly from a random distribution. A Watson-Williams F-test was used to compare mean orientation vector angles (i.e. degrees) among treatment groups and trials. All values are reported as mean ± s.e.m. and tests were deemed significant at $\alpha < 0.05$.

3.4 Results

3.4.1 Morphological measurements

The most parsimonious models for body mass (Table S3.4), fat score (Table S3.5), and moult score (Table S3.6) indicated photoperiod, treatment, and sex to be important predictors. There was a significant curvilinear increase in mean body mass of all birds over the photoperiod shift between 15L:09D and 09L:15D (β ± S.E.: 0.042 ± 0.0091, $p < 0.001$; Figure 3.1*a* , Table

S3.4). Fat scores showed a linear increase between 15L:09D and 09L:15D (β ± S.E.: 0.23 ± 0.062, $p < 0.001$; Figure 3.1*b*, Table S3.5). Sex influenced body mass (β ± S.E.: 0.092 ± 0.032, $p = 0.01$; Table S3.4) but not fat score: males from all treatment groups were significantly heavier than females. There were no significant effects of treatment on fat score, but birds exposed to the high treatment group had significantly greater body mass than other treatment groups (β ± S.E.: 0.071 ± 0.033, $p = 0.037$; Table S3.4). All birds had initiated their moult prior to the start of the photoperiod shift; however, there was a significant curvilinear increase in moult score of all birds over the entire photoperiod shift, levelling off after 12L:12D (Photoperiod: β ± S.E.: 0.20 ± 0.051, $p < 0.001$; Figure 3.1*c*, Table S3.6). Total moult score showed no significant differences between treatment groups (β ± S.E.: -0.45 ± 0.36, $p = 0.22$; Figure 3.1*c*, Table S3.6). Figure 3.2 however, indicates that high dosed birds tended to show a delay in completion of moult relative to the controls. Upon further analysis, this delay in moult completion was attributed to a sex effect, with a two-way significant interaction existing between treatment and sex for moult score (β ± S.E.: -0.28 ± 0.12, $p = 0.024$; Table S3.6). Males from the high treatment group had a moult score that was significantly lower than controls ($\chi^2 = 7.12$, df = 1, $p = 0.01$; Figure 3.2), while there were no significant differences in the primary moult score of female starlings among treatments. In the high treatment group, males had lower primary moult scores than females ($\chi^2 = 5.42$, df = 1, $p = 0.02$; Figure 3.2). There were no interactions between treatment and photoperiod/time in any of the tested models (Tables S3.4-S3.6).

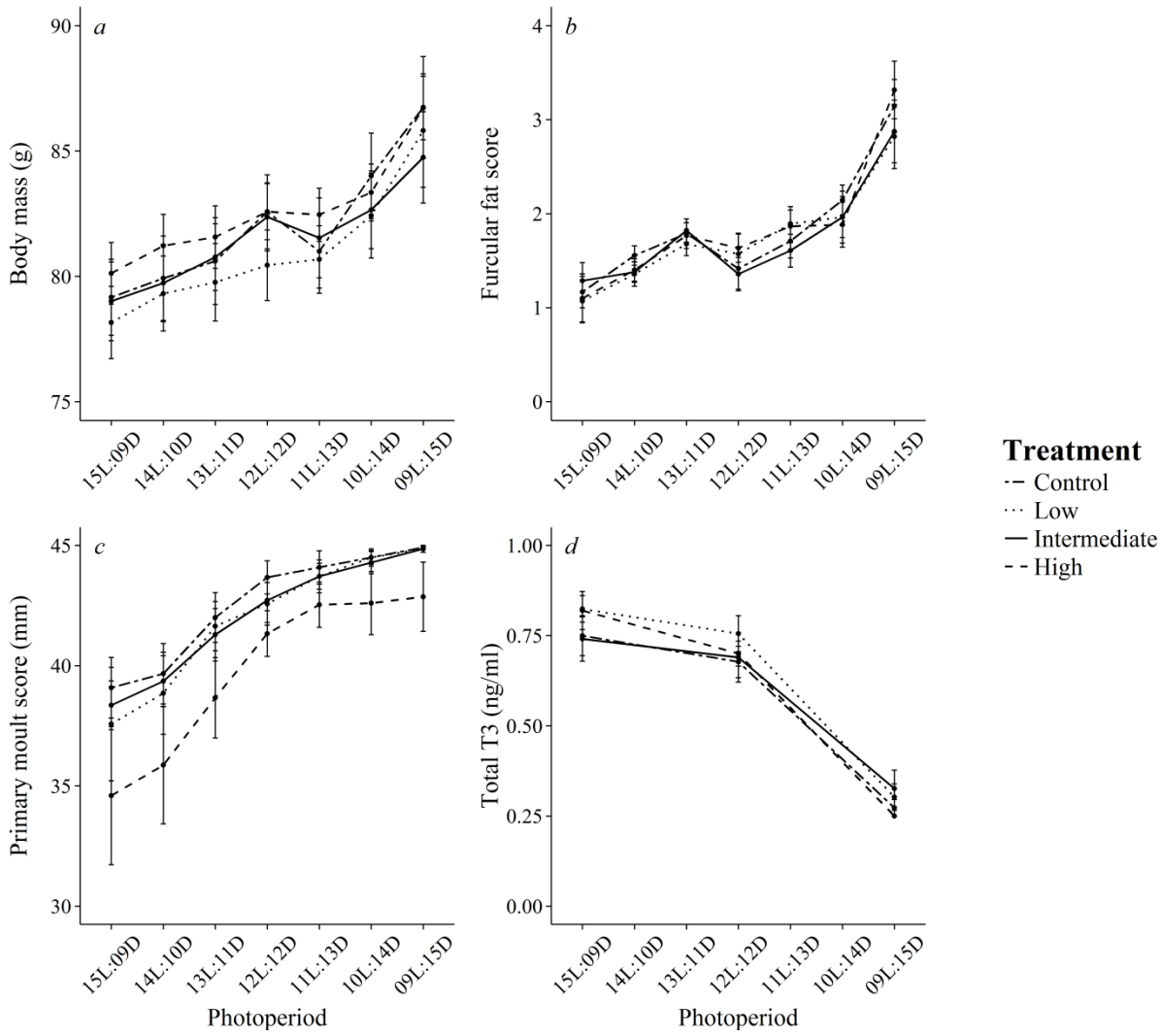

Figure 3.1. (*a*) Body mass (g), (*b*) furcular fat score, (*c*) moult score (mm), and (*d*) plasma total triiodothyronine (T3) (ng/ml) measured over a 6-week photoperiod shift simulating autumn migration in juvenile European starlings exposed to 0 (control), 0.35 (low), 0.70 (intermediate), or 1.05 (high) µg Aroclor 1254/g-bw/day. Data represented as mean ± s.e.m. There were no significant differences in body mass, fat score, moult score, or T3 levels among treatments.

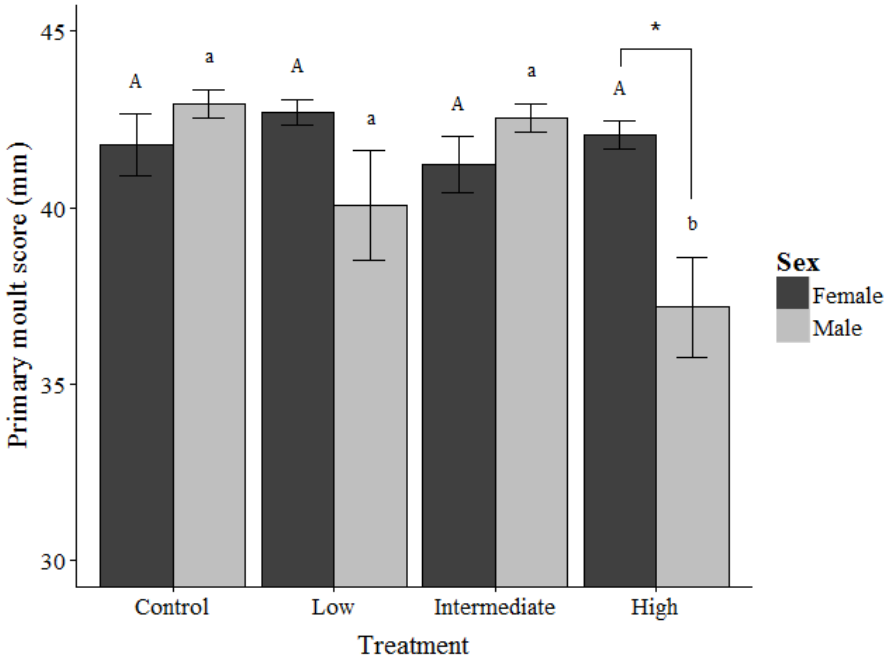

Figure 3.2. Primary moult score measured over a 6-week photoperiod shift simulating autumn migration in juvenile European starlings exposed to 0 (control), 0.35 (low), 0.70 (intermediate), or 1.05 (high) µg Aroclor 1254/g-bw/day. Data represented as mean ± s.e.m. Different letters (uppercase = female, lowercase = male) indicate a significant difference ($p < 0.05$) in primary moult score compared to controls of same sex. Significant differences between males and females are indicated as: * $p < 0.05$.

3.4.2 Thyroid hormones

Plasma total T3 (ng/ml) of all birds showed a significant curvilinear decrease over the duration of the photoperiod shift (Figure 3.1*d*, Table S3.7). Plasma total T3 was not affected by treatment or sex (Table S3.7). Plasma total T4 values were all below the levels of detection of the assay (1.28 ng/ml).

3.4.3 Migratory activity and orientation

A strong positive correlation between the number of hops in each funnel and the total measured activity of each bird (r = 0.855, df =265, $p < 0.001$) suggested both measures were good predictors of migratory activity. Migratory activity was best predicted by treatment group, photoperiod, and sex. Activity levels across all birds showed a variable response over time, peaking at 12L:12D and reaching their lowest levels at the end of the experiment under winter daylengths of 09L:15D (Figure 3.3, Table S3.8). Migratory activity did not differ significantly among treatment groups; however, there was a trend towards decreasing activity in birds exposed to the intermediate treatment group (β ± S.E.: -1.22 ± 0.66; Table S3.8).

Starlings showed a preferred orientation during all trials; however, directional vectors were not consistent between the measured photoperiods (Table 3.1). At 12L:12D, during the peak of migratory activity, birds from the control, low, and intermediate treatment groups demonstrated a directional preference (Rao's U = 177.1, n = 12, r = 0.17, $p < 0.05$), while birds from the high treatment group demonstrated a random distribution (no orientation) (Rao's U = 141.0, n = 14, r = 0.25, $p > 0.05$; Figure 3.4). While there were no significant differences in mean orientation vector among treatment groups (Watson-Williams F = 1.17, n = 53, p = 0.33), control birds (0 ppm) oriented to 155.95°(South-southeast), while the remaining treatment groups varied in their direction of orientation (Figure 3.4). High-dosed birds were subsequently delayed in showing a preferred orientation until 10L:14D (2 weeks later), where they oriented to 197.48° (South-southwest).

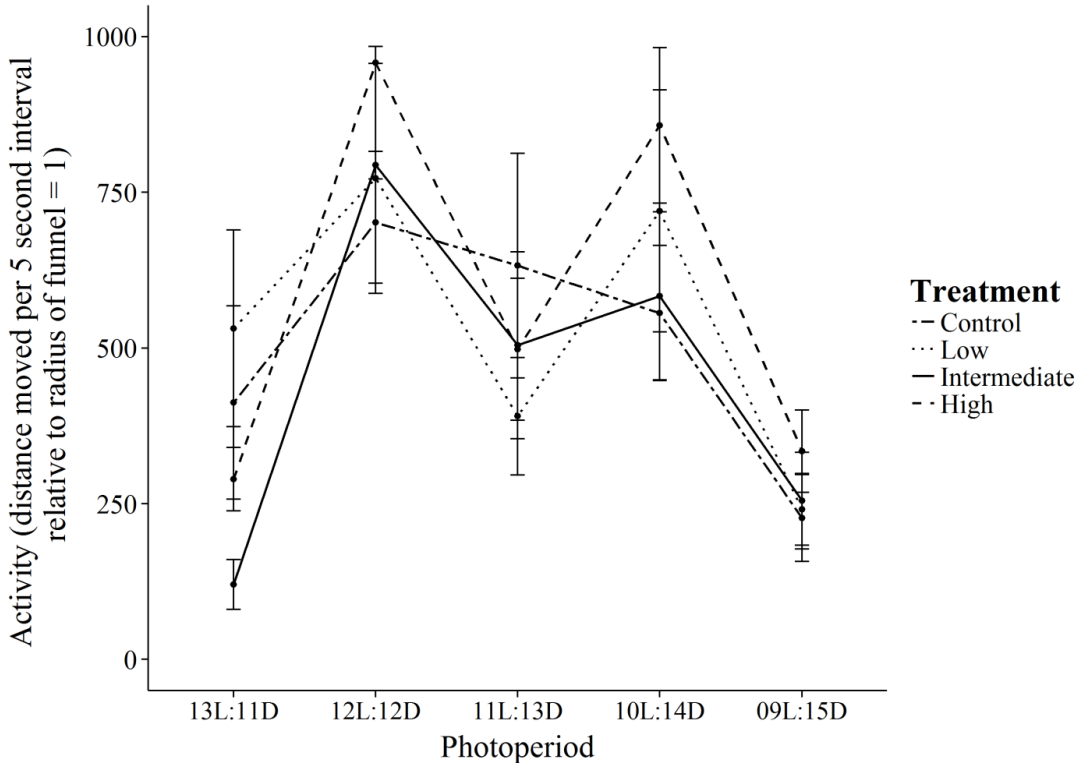

Figure 3.3. Total mean migratory activity over a 6-week photoperiod shift simulating autumn migration in juvenile European starlings exposed to 0 (control), 0.35 (low), 0.70 (intermediate), or 1.05 (high) ug Aroclor 1254/g-bw/day. Data represented as mean ± s.e.m. Observations of birds were recorded in the Emlen funnel for 25 minutes, after a 5 minute acclimation period.

Table 3.1. Orientation of birds under each photoperiod and treatment level. Asterisks (*) indicate that the bird demonstrated a significant directional preference (p = significance level according to the Rao's spacing test; **p < 0.01, *p < 0.05, N.O = no orientation; p > 0.05). Shading indicates the peak in autumn migratory activity observed across treatment groups at 12L:12D photoperiod cycle. n = sample size; α (°) = mean vector orientation (deg).

		treatment											
		control			**low**			**intermediate**			**high**		
		n	**α (°)**	**compass direction**	***n***	**α (°)**	**compass direction**	***n***	**α (°)**	**compass direction**	***n***	**α (°)**	**compass direction**
photoperiod	**13L:11D**	11	n.o	-	12	n.o	-	12	n.o	-	14	307**	NW
	12L:12D	**12**	**156***	**SSE**	**13**	**92***	**E**	**14**	**120***	**ESE**	**14**	**N.O**	**-**
	11L:13D	12	n.o	-	14	282**	WNW	14	n.o	-	15	298*	WNW
	10L:14D	12	n.o	-	14	n.o	-	14	n.o	n.o	15	198*	SSW
	09L:15D	12	334**	NNW	14	327**	NNW	14	333*	NNW	15	337**	NNW

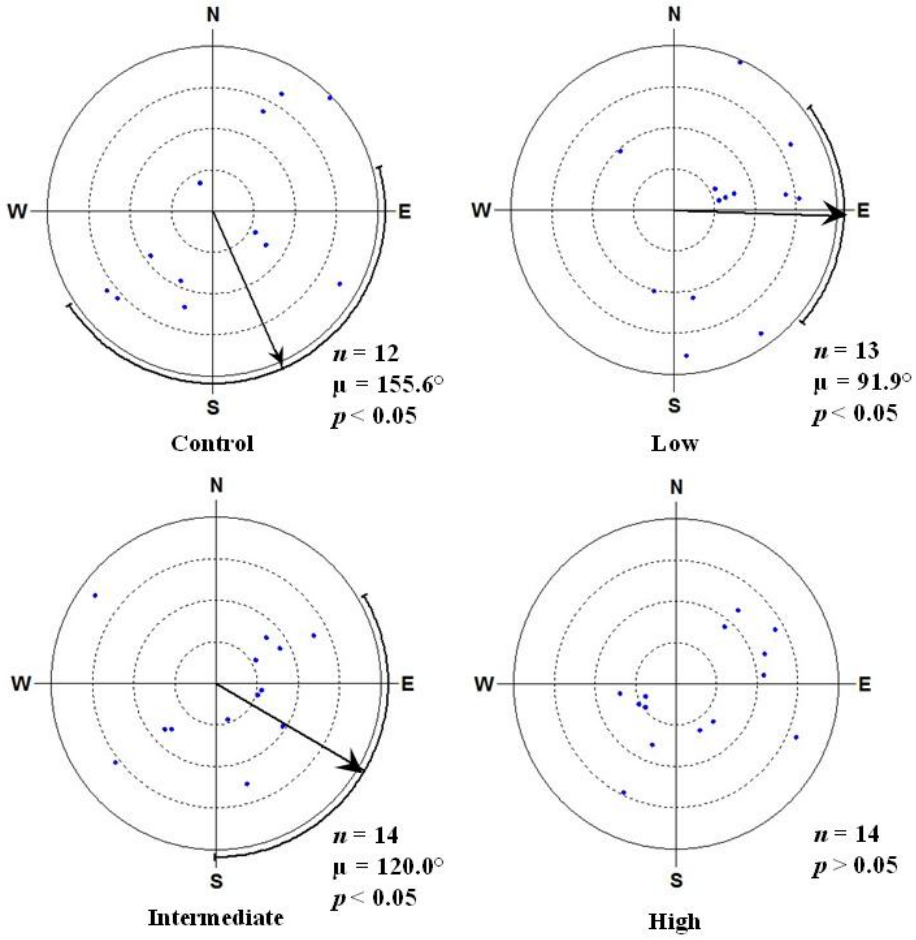

Figure 3.4. Mean orientation direction and magnitude of migratory active European starlings under a 12L:12D photoperiod in modified Emlen funnels. Each dot represents the mean bearing and magnitude (distance from centroid) of one bird. The arrow direction indicates the migratory vector orientation (mean angle, θ) for each treatment group. Birds from control, low, and intermediate treatments demonstrated a preferred (unimodal) orientation (Rao's spacing test; $p < 0.05$) with outer curve showing confidence intervals, while birds from high treatment group demonstrated a random orientation (Rao's spacing test; $p > 0.05$).

3.5 Discussion

To the best of our knowledge, this is the first study demonstrating a link between early exposure to EDCs and latent alterations in migratory behaviour. Although migratory activity was induced under captive conditions, there was a significant delay in the ability of PCB-exposed European starlings to correctly orient under simulated autumn migratory conditions. We also found that there was a significant delay in completion of primary moult in male starlings exposed to 1.05 µg Aroclor 1254/g-bw/day (high treatment group) compared to untreated males. This suggests that early PCB exposure may have 1) caused delays in ontogeny related to moult and migratory events and/or 2) impaired neuroendocrine and/or neurocognitive function relating to orientation and navigation. In exploring these potential, and non-mutually-exclusive, hypotheses, the potential role of thyroid hormone homeostasis during migration as a mechanism for controlling these key life cycle events was examined.

3.5.1 Impacts of PCB exposure on the ontogeny of a migration event

Regulation of the timing and manifestation of avian reproduction, moult, and migration in many passerine species is controlled by multiple environmental inputs, primarily seasonal changes in daylength, and intrinsic factors such as endogenous circadian and circannual rhythms within the bird[57, 59]. We found that as photoperiod decreased from 13h to 12h of daylight, there was a significant increase in the activity of birds tested in funnels, followed by a continued decrease as photoperiod reached short winter daylengths. This development of migratory restlessness was accompanied by an increase in body mass and furcular fat deposits, supporting our previous findings that this photoperiod (12L:12D) corresponds to the peak of migratory

activity in this passerine species at this latitude[59, 117], and suggests that birds from all treatments were responding similarly to the change in light regime.

Consistent with the changes in activity, mass, and moult, all treatment groups showed a significant decrease in thyroid hormone concentrations as the photoperiod was shifted from long to short daylengths; however, we found no treatment-related effects on circulating levels of T3 throughout the experiment. Both T3 and T4 are important for the entire complement of events associated with migration[70], directly influencing the initiation of moult and migration, along with the development of premigratory fattening and migratory restlessness[68, 70]. While there was an overall acceleration in the completion of moult for all starlings over the duration of the photoperiod shift, male birds from the high treatment group were markedly delayed in this completion and on average, had not completed a full moult by the completion of the experiment. High-dose males had a significantly lower moult score when compared to untreated males, and when compared to females from the same treatment. These results are consistent with a study showing a dose-dependent delay in moult progression of goldfinches (*Carduelis tristis*) during a 4-month daily exposure to environmentally relevant levels of linuron (0 - 16 µg/g-bw/d), a herbicide with anti-androgenic and anti-thyroid activity[6]. Birds from the medium and high treatment groups demonstrated a delay in moult peak, which was mainly attributed to depressed levels of plasma T4[118]. The key difference between these results and the current study is that a delay in moult progression of high-dose starlings was exhibited almost 4 months after dosing had been completed, indicating that latent effects resulting from an earlier exposure to EDCs did persist into adulthood. Unfortunately, in the present study, we were unable to quantify plasma T4 concentrations due to the detection limit of the assay, nor did we measure other candidate hormones involved in moult such as corticosterone or prolactin, which could contribute to

observed delays in male moult. Disruption of thyroid hormones and other endocrine regulators of moult and feather regrowth may indicate effects of an exposure to EDCs that persist into adulthood[118, 119]. Alterations in thyroid hormone function could have direct effects on both initiation and progress of moult in migratory birds, along with carry-over effects on success of migration, breeding, and fitness. Further exploration into the mechanistic underpinnings of thyroid hormone disruption during feather development is needed to better understand the significant delays in moult completion observed in the present study.

3.5.2 Impacts of EDCs on avian neuroendocrine function

It has been estimated that over one million tons of PCBs were produced worldwide between 1930 and 1993[12]. Though since banned, PCBs can still be released into the environment through improper waste management, disposal strategies, and leakage[14], and consequently occur at detectable concentrations in virtually every level of the global ecosystem[2]. There is widespread evidence suggesting that early exposure to PCBs and other similar dioxin-like compounds can result in a range of impacts on individuals and populations of birds[22, 34, 39], frequently through alterations in thyroid hormone levels[19, 34]. Birds exposed to sublethal concentrations of PCBs in heavily contaminated sites have exhibited alterations in song performance[120], lower nest quality[121], impaired reproductive success[122], and decreased chick survival[22]. The treatments chosen for this study represent exposure concentrations of PCBs representative of contamination at the lower spectrum of PCB exposure. Exposure to these low concentrations have also produced measurable results in avian species, including effects on growth, immune function, song output, and behaviours related to breeding and reproduction[22, 32, 33, 97]. There are a suite of developmental effects that can occur, such as embryonic mortality, teratogenicity, decreased hatching and fledgling success, decreased growth rates, and

immunotoxicity[39, 51], all of which can have both immediate and long-lasting effects on chick viability and survival. Some of the more pronounced behavioural alterations arising from exposure to PCB mixtures have included abnormal parenting behaviour such as inadequate incubation and nest defence[108], reduced nest attentiveness[109], and altered courtship behaviour[110]. Further examination of the relationship between PCB exposure and altered long-term developmental processes in birds is crucial for understanding the potential for latent effects on behaviours and life history events that are critical for survival and reproduction[111]. Increasingly, researchers have recognized the potential for early alterations in thyroid hormone homeostasis and consequent brain morphology to result in long-term and irreversible changes in brain function, cognition, and behaviour[1]; however, there is a general lack of information regarding the impact of EDCs on development of brain regions related to avian migration. Although our study has no direct evidence supporting this, the neurodevelopmental toxicity of PCBs is known to arise from interaction with different processes of brain development, but interference with hormone signalling in the developing brain has been shown to be a predominant pathway through which disruption of thyroid function occurs[36]. Disruption of these essential processes may indicate effects of exposure to PCBs that persist into adulthood, making the brain highly susceptible to embryonic and post-hatch EDC exposure[47]. Furthermore, the risk of exposure and sensitivity to PCBs and OH-PCBs, in addition to other structurally similar compounds (e.g. polybrominated diphenyl ethers), during critical windows of embryonic and nestling development is likely greater than that of adults because young individuals undergo large and rapid structural and functional changes, making them particularly vulnerable to any long-term toxic effects of chemicals[51].

In previous displacement experiments during autumn migration events[123], juvenile starlings embarking upon their first migration revealed different orientation strategies compared to adult migrants. Juvenile starlings possess an innate knowledge of the migratory direction to their wintering grounds and by using a 'bearing-and-distance' program (clock and compass orientation), flying in a fixed direction and distance without specific knowledge about the landmarks of their goal[124]. Juveniles use environmental references such as the earth's geomagnetic field, the stars, and/or the sun to obtain a direction of reference[125, 126], whereas adults use true goal orientation (homing orientation). The mean migratory orientation (SSE) of control starlings measured under autumn photoperiod conditions (12L:12D) corresponds to that of free-flying local starling migrants[127]. This suggests that examining migratory restlessness and orientation of birds exposed to a 12L:12D photoperiod was the most useful and relevant time period when assessing the effects of an exposure to EDCs on these parameters that directly influence the success of an avian migration event. Equally, because juveniles are using innate orientation, we believe that impairment suggests PCBs are more likely to have affected those brain regions involved in spatial orientation, rather than memory.

The avian hippocampus plays an important role in spatial processing, learning, and memory[128, 129], which are essential elements in the brain regions involved with navigation[130, 131] and creation of a spatial map in migratory birds[132]. The hippocampus is a structure sensitive to alterations in hormone levels, including thyroid hormones[133], and so further research is needed to elucidate potential alterations in the hippocampi of birds that are impaired in their orientation or navigational abilities. Hippocampal lesions in homing pigeons have resulted in an impaired ability to navigate towards unfamiliar locations, suggesting that any hormone-induced structural

changes in the hippocampus could result in the inability of a juvenile passerine to utilize an internal 'sun or magnetic compass' during development of a cognitive migration map[134].

3.5.3 Relevance to wild bird populations

In the current study it has been demonstrated that juvenile starlings treated with 1.05 µg Aroclor 1254/g-bw/day displayed a preferred orientation in four out of the five trials (Table 3.1); however, their orientations ranged from NNW to WNW - directions that are incorrect based on typical migratory patterns of passerines at this latitude[127]. The only photoperiod where high-dose birds failed to orient was at 12L:12D, which corresponded to the peak of migratory activity and correct orientation in control birds. It was not until two weeks after the critical time period for migratory activity and orientation (10L:14D) that birds from the high treatment group oriented SSW, indicating that they were significantly delayed in their ability to correctly orient. These results, combined with the significant absence of moult completion in birds from the high treatment group, suggest that these birds would be at a distinct disadvantage in the wild when compared to non-exposed individuals. Alterations in the timing and accuracy of migratory events are likely to be maladaptive and can have significant consequences for wild bird populations. Birds can encounter ecological barriers and unsuitable habitats during migration, resulting in burdens on their energy and water budgets[114], making it critical to correctly orient and navigate to essential stopover sites, wintering, or breeding grounds. Prolonged migration or delays in departure to wintering/breeding grounds resulting from early EDC exposure can result in negative carry-over effects such as delayed reproduction, poorer annual survival, reduced food supplies at stopover sites, higher energy expenditure, and increased chances for moving off-course of an established route[135]. Migratory birds that have sufficient fat and body mass reserves but are significantly delayed in their completion of moult and/or orientation ability could be at a

distinct disadvantage when considering the intense amount of competition for resources at stopover sites and breeding grounds[136].

Migratory birds must also maintain long-term memories associated with stopover sites, wintering grounds, and breeding sites, implying that birds have evolved advanced and innate cognitive abilities required to successfully orient and navigate as juveniles[125]. Although European starlings serve as highly useful models for studies examining ecological effects of exposure to environmental contaminants[22], they are considered short-distance diurnal migrants that exhibit migration characteristics such as flocking behaviour, intermittent short-hops along the route, and reliance upon multiple cues for successful migration[114]. By comparison to some other long-distance, solitary, nocturnal migrants, the starling's life history characteristics likely contribute to their adaptability and success as a population. However, other migratory species could be more strongly affected by developmental effects on endocrine processes affecting migration.

Some well-known causes of vagrancy in birds include wind drift, autumn 180° orientation (reverse migration), and spring overshooting[137, 138]. While weather patterns have been documented to play a role in occurrences of vagrant birds, the degrees to which weather or other factors influence vagrancy[137] are not well understood. Vagrant birds typically originate from migratory populations and are primarily inexperienced juveniles on their first autumn migration[124], prompting questions as to whether unfavourable weather conditions alone are the cause of vagrancy in juveniles. When confronted with displacement along a migration route, misoriented juveniles cannot perform navigational corrections, decreasing the likelihood of their arrival at a location suitable for resting or wintering. Any errors in navigation, combined with the intense selection pressures that birds face during migration, could result in deleterious

consequences for subsequent survival and breeding success[136]. Ultimately, even subtle alterations in the physiological and behavioural mechanisms required for a successful migration can have potentially negative consequences for population viability[112].

3.6 APPENDIX

Additional descriptions of chemical analyses, general linear mixed model (GLMM) statistical model output, and Emlen funnel trial sample sizes are provided.

Materials and Methods

Extraction and clean-up of dosing solutions

HPLC grade Acetone and Hexane, anhydrous sodium sulfate (certified ACS Granular) were purchased from Fisher Scientific (Fair Lawn, NJ, USA), Silica gel (pore size 60Å, 60-100 mesh, high-purity Davisil Grade 635) from Sigma-Aldrich (St. Louis, MO, USA). PCB standards were purchased from Wellington Laboratories, and included 27 mass labelled recovery and 5 mass labelled internal standards, a full list of native and mass labelled PCBs are given in the supporting materials (Table S2.1). Vials used in extraction were purchased from Chromspec (Chromatographic Specialties Inc., Brockville, ON, CA)

Dosing solution (100 µl) extractions were carried out following a method adapted from EPA Method 1668B (US-EPA 2008)[1]. All samples were fortified with 100ng/ml surrogate standard and extracted and cleaned using a multi-layer silica column (1g NaSO4, 2g basic silica (23% NaOH), 1g NaSO4, 4g acid Silica (30% sulphuric acid), 1g NaSO4), preconditioned with 150ml of *n*-Hexane. Samples were added to the column and were eluted with 250mL of *n*-hexane, collected in a round bottom flask, and rotary evaporated to 1ml. Extracts were transferred to 5ml vials with washings and concentrated under nitrogen to ~100 µl, and transferred with washings to GC vials and solvent exchanged to 10 µl of nonane with 100ng/ml labelled internal standard.

Identification and quantification of all target compounds was performed by GC/MS using an Agilent 7890A gas chromatograph equipped with an HT8 column (60 m x 0.25 mm i.d., 0.25 µm film thickness), connected to a 5975C mass spectrometer detector (MSD) operating using EI in SIM mode, (Agilent Technologies, Wilmington, DE, USA. Two µl were injected in splitless mode with an injector temperature of 250°C, and a helium flow of 1.5ml min^{-1}, GC oven temperature was initially 100°C held for 2 min, heated to 140°C at 20°C/min, to 200°C at 4°C/min, then to 300°C at 4°C/min and held for 17.5 min, the transfer line was maintained at 300°C. Aroclor concentrations were based on the concentrations of an Aroclor 1254 standard diluted in a 5-point calibration from 1 ng/ml to 2000 ng/ml. The R^2 of the native PCBs in the calibration curve was calculated to be > 0.99. Final concentrations for dosing solutions of Aroclor 1254 are expressed as µg Aroclor 1254/ml (ppm) and µg Aroclor 1254/g tissue (ppm).

Quality Assurance and Quality Control (QA/QC)

All equipment used was pre-cleaned with acetone and *n*-hexane to avoid sample contamination during cleanup, extraction, and chemical analysis, and where possible vials were baked at 450°C before use. A procedural blank was included for every 10 samples and was found to be 0 ng/g, indicating that there was no sample contamination.

Recovery concentrations ranged from 1.9% to 39.6% with a mean of 14.5%, the MDL was calculated as 3X the standard deviation plus the mean of blanks for each congener and in general the samples were above this indicating limited contamination during extraction.

Hexane blanks were included during each GC/MS run after every 6 samples to assess carryover and check the column condition. An internal standard was included after every 12 samples to

check retention times for shifts throughout the chromatogram and ensure correct PCB peak identification, any drift seen during a run and the samples were stopped.

References

1. US EPA 2008. 2008 Method 1668B: Chlorinated Biphenyl Congeners in Water, Soil, Sediment, Biosolids, and Tissue by HRGC/HRMS. Washington (DC): Engineering and Analysis Division, Office of Science and Technology, Office of Water, U.S. Environmental Protection Agency, 1-128.

Table S3.1. Native and mass labelled PCBs and the ions (m/z) used to monitor each.

	12C		**13C**	
Mono	188	190	200	202
Di	222	224	234	236
Tri	256	258	268	270
Tetra	290	292	302	304
Penta	324	326	337	339
Hexa	360	362	372	374
Hepta	394	396	406	408
Octa	428	430	440	442
Nona	462	464	474	476
Deca	496	500	508	510

Table S3.2. Nominal and measured concentrations (µg/ml) of Aroclor 1254 in each treatment and stock solution. Data represented as mean ± s.e.m. Significant differences between control and treatments are indicated as: * $p < 0.05$.

Nominal concentration µg/ml (ppm)	**Measured concentration (µg/ml (ppm))**
0 (control)	0.032 ± 0.00
50 (low)	51.72 ± 0.022
100 (intermediate)	85.04 ± 0.015
150 (high)	153.14 ± 0.44*
1000 (stock)	987.47 ± 1.85

Table S3.3. Number of birds tested in Emlen funnels per treatment level, sex, and photoperiod. *n* = sample size.

Treatment	**Sex**	**photoperiod**				
		13L:11D	**12L:12D**	**11L:13D**	**10L:14D**	**09L:15D**
control	males	8	8	8	8	8
	females	3	4	4	4	4
low	males	3	4	4	4	4
	females	9	9	10	10	10
intermediate	males	7	9	9	9	9
	females	5	5	5	5	5
high	males	6	6	7	7	7
	females	8	8	8	8	8
***n* tested per week**		**49**	**53**	**55**	**55**	**55**

Table S3.4. Results of general linear mixed model analyzing body mass in response to treatment, sex, and photoperiod. Body mass measured over a 6 week photoperiod shift simulating an autumn migration in 55 juvenile European starlings exposed to 4 concentrations of Aroclor 1254/g-bw/day.

Fixed Effects	**β Estimate ± SE**	**t**	***p***	**df**
(Intercept)	4.26 ± 0.031	139.52	< 0.001	323
Treatment (Reference: 0 ppm)				
50ppm	0.041 ± 0.031	1.32	0.20	33
100ppm	0.051 ± 0.036	1.40	0.17	33
150ppm	0.071 ± 0.033	2.17	0.04	33
Sex	0.092 ± 0.032	2.90	0.01	33
Photoperiod	0.042 ± 0.0091	4.55	< 0.001	323
$Photoperiod^2$	-0.010 ± 0.0024	-4.32	< 0.001	323
$Photoperiod^3$	0.00099 ± 0.0002	4.96	< 0.001	323
Treatment x Sex (Reference: 0ppm Female)				
50ppm Male	-0.026 ± 0.039	-0.68	0.50	33
100ppm Male	-0.051 ± 0.044	-1.16	0.25	33
150ppm Male	-0.059 ± 0.039	-1.52	0.14	33
Treatment x Photoperiod (Reference: 0ppm 09D)				
50ppm Photoperiod	-0.000029 ± 0.0037	-0.0078	0.99	323
100ppm Photoperiod	-0.0031 ± 0.0036	-0.85	0.40	323
150ppm Photoperiod	-0.0024 ± 0.0036	-0.68	0.50	323
Sex x Photoperiod (Reference: Female 09D)	-0.0013 ± 0.0026	0.53	0.60	323

Random Effects	**Variance**	**SD**
Nestbox (Intercept)	0.0017	0.041
BirdID (Intercept)	0.0032	0.057
Photoperiod (Slope)	6.7×10^{-5}	0.0082

Table S3.5: Results of general linear mixed model analyzing furcular fat score in response to treatment, sex, and photoperiod. Furcular fat score (0-5) measured over a 6 week photoperiod shift simulating an autumn migration in 55 juvenile European starlings exposed to 4 concentrations of Aroclor 1254/g-bw/day.

Fixed Effects	**β Estimate ± SE**	**t**	***p***	**df**
(Intercept)	0.7 ± 0.28	2.67	0.01	325
Treatment (Reference: 0 ppm)				
Low	-0.082 ± 0.33	0.25	0.81	33
Intermediate	0.19 ± 0.37	0.51	0.61	33
High	-0.0017 ± 0.34	-0.05	0.96	33
Sex	0.11 ± 0.32	0.35	0.73	33
Photoperiod	0.23 ± 0.062	3.69	< 0.001	325
Treatment x Sex (Reference: 0ppm Female)				
Low Male	-0.22 ± 0.40	-0.54	0.59	33
Intermediate Male	-0.12 ± 0.40	-0.3	0.77	33
High Male	-0.084 ± 0.38	-0.22	0.83	33
Treatment x Photoperiod (Reference: 0ppm 15L:09D)				
Low Photoperiod	-0.00054 ± 0.074	-0.0074	0.99	325
Intermediate Photoperiod	-0.046 ± 0.071	-0.65	0.52	325
High Photoperiod	0.031 ± 0.071	0.43	0.66	325
Sex x Photoperiod (Reference: Female 15L:09D)	0.032 ± 0.051	0.63	0.53	325

Random Effects	**Variance**	**SD**
Nestbox (Intercept)	0.011	0.1
BirdID (Intercept)	0.17	0.41
Photoperiod (Slope)	0.02	0.14

Table S3.6: Results of general linear mixed model analyzing right wing chord moult score in response to treatment, sex, and photoperiod. Total moult score measured over a 6 week photoperiod shift simulating an autumn migration in 55 juvenile European starlings exposed to 4 concentrations of Aroclor 1254/g-bw/day.

Fixed Effect	**β Estimate ± SE**	**t**	***p***	**df**
(Intercept)	6.13 ± 0.32	19.43	< 0.001	324
Treatment (Reference: 0 ppm)				
50ppm	-0.25 ± 0.37	-0.67	0.51	33
100ppm	-0.062 ± 0.36	-0.17	0.86	33
150ppm	-0.45 ± 0.36	-1.26	0.22	33
Sex	-0.25 ± 0.27	-0.93	0.36	33
Photoperiod	0.20 ± 0.051	3.98	0.0001	324
Photoperiod^2	-0.019 ± 0.0030	-6.28	< 0.001	324
Treatment x Sex (Reference: 0ppm Female)				
50ppm Male	-0.0051 ± 0.13	-0.04	0.97	33
100ppm Male	-0.014 ± 0.12	-0.11	0.91	33
150ppm Male	-0.28 ± 0.12	-2.37	0.024	33
Treatment x Photoperiod (Reference: 0ppm 09D)				
50ppm Photoperiod	0.041 ± 0.053	0.78	0.44	324
100ppm Photoperiod	0.0080 ± 0.051	0.16	0.88	324
150ppm Photoperiod	0.075 ± 0.051	1.47	0.14	324
Sex x Photoperiod (Reference: Female 09D)	0.045 ± 0.037	1.23	0.22	324

Random Effects	**Variance**	**SD**
Nestbox (Intercept)	1.54×10^{-8}	0.00012
BirdID (Intercept)	0.77	0.88
Photoperiod (Slope)	0.015	0.12

Table S3.7: Results of general linear mixed model analyzing plasma total triiodothyronine (T3) (ng/ml) in response to treatment, sex, and photoperiod. Total mean T3 (ng/ml) measured over a 6 week photoperiod shift simulating an autumn migration in 55 juvenile European starlings exposed to 4 concentrations of Aroclor 1254/g-bw/day.

Fixed Effects	**β Estimate ± SE**	**t**	***p***	**df**
(Intercept)	-0.41 ± 0.13	-3.15	0.0022	84
Treatment (Reference: 0 ppm)				
50ppm	0.094 ± 0.14	0.66	0.52	33
100ppm	0.065 ± 0.16	0.41	0.69	33
150ppm	0.060 ± 0.15	0.41	0.69	33
Sex	-0.074 ± 0.14	-0.55	0.59	33
Photoperiod	0.18 ± 0.039	4.55	< 0.001	84
Photoperiod^2	-0.044 ± 0.0044	-10.26	< 0.001	84
Treatment x Sex (Reference: 0ppm Female)				
50ppm Male	-0.011 ± 0.17	-0.064	0.95	33
100ppm Male	-0.18 ± 0.17	-1.034	0.31	33
150ppm Male	0.065 ± 0.16	0.4	0.69	33
Treatment x Photoperiod (Reference: 0ppm 09D)				
50ppm Photoperiod	-0.00043 ± 0.021	-0.021	0.98	84
100ppm Photoperiod	0.019 ± 0.021	0.93	0.36	84
150ppm Photoperiod	-0.019 ± 0.020	-0.98	0.33	84
Sex x Photoperiod (Reference: Female 09D)	0.017 ± 0.015	1.15	0.26	84

Random Effects	**Variance**	**SD**
Nestbox (Intercept)	1.54×10^{-4}	0.012
BirdID (Intercept)	0.024	0.16
Photoperiod (Slope)	-	-

Table S3.8: Results of general linear mixed model analyzing activity in response to treatment, sex, and photoperiod. Total mean migratory activity measured over a 6 week photoperiod shift simulating an autumn migration in 55 juvenile European starlings exposed to 4 concentrations of Aroclor 1254/g-bw/day.

Fixed Effects	**β Estimate ± SE**	**t**	***p***	**df**
(Intercept)	1.17 ± 0.83	1.4	0.16	99
Treatment (Reference: 0 ppm)				
50ppm	-0.31 ± 0.63	-0.5	0.62	99
100ppm	-1.22 ± 0.66	-1.84	0.07	99
150ppm	-0.39 ± 0.62	-0.63	0.53	99
Sex	-0.080 ± 0.51	-0.16	0.88	99
Photoperiod	1.92 ± 0.37	5.25	< 0.001	55
Photoperiod^2	-0.25 ± 0.043	-5.85	< 0.001	55
Treatment x Sex (Reference: 0ppm Female)				
50ppm Male	0.80 ± 0.43	1.86	0.07	99
100ppm Male	0.51 ± 0.51	1.01	0.32	99
150ppm Male	0.52 ± 0.44	1.18	0.24	99
Treatment x Photoperiod (Reference: 0ppm 09D)				
50ppm Photoperiod	0.0056 ± 0.14	0.04	0.97	55
100ppm Photoperiod	0.16 ± 0.14	1.17	0.25	55
150ppm Photoperiod	0.12 ± 0.13	0.87	0.39	55
Sex x Photoperiod (Reference: Female 09D)	-0.036 ± 0.098	-0.37	0.72	55

Random Effects	**Variance**	**SD**
Nestbox (Intercept)	0.096	0.31
BirdID (Intercept)	0.0018	0.043
Camera Position (Intercept)	0.35	0.6
Subtrial (Intercept)	0.33	0.58

4 CONCLUSIONS

4.1 Development of a migratory passerine songbird model and test system for studying effects of an endocrine disrupting compound on migration

A key component of this study was the development of an appropriate model species and test system for understanding the effects of contaminants on migratory behaviour. Findings from pilot studies in 2011 using 2 species (European starling and red winged blackbird) indicated that the European starling was an excellent model for linking early exposure to an endocrine disrupting mixture to latent and long-term effects on the critical life-stage of migration. Starlings readily take up nestboxes to breed and will nest in loose colonies, which is optimal for reducing risk of predation and providing a suitable sample size. They were amenable to captivity and exhibited predictable changes in body mass, fat accumulation, moult, and increased activity under simulated shifts in photoperiod. Both species had little or no mortality associated with exposure to Aroclor 1254; however, red-winged blackbirds did exhibit greater natural mortality both in the field and in captivity when compared to starlings. Field mortality in red-winged blackbirds can likely be attributed to two causes: 1) the open nest utilized by red-winged blackbirds and 2) unintentional creation of corridors in the wetland through repeated visits to nest. Although well concealed in wetlands, open-cup and group nesting strategies employed by red-winged blackbirds, combined with the potential for corridors, could have contributed to disappearances of nestlings. Although both starlings and red-winged blackbirds were observed to reach independence in captivity by roughly 30 days, red-winged blackbirds fledge much earlier from the nest at 10 – 12 days such that they required a longer period of care and labour-intensive feeding in captivity.

Our exposure technique involved daily oral gavage, which was confirmed through measuring daily body mass of nestlings and obtaining dosing volumes corrected for body mass. While other studies have successfully employed the approach of injecting mealworms with known concentrations of chemicals and feeding to songbirds such as the European starling[32, 33], this relies upon a number of assumptions, such as calculations of daily food intake, dietary absorption efficiencies, and toxicokinetics following feeding[139]. By administering a consistent dose of Aroclor 1254 dissolved in an oil vehicle via oral gavage, we increased the speed and ease of dosing and minimized uncertainties associated with calculations.

We also demonstrated that photoperiodic manipulations in captivity can successfully induce migratory activity of starlings. However, red-winged blackbirds did not demonstrate the same pattern and did not show signs of migratory restlessness when trialed. Similar to a study of the orientation behaviour in chaffinches (*Fringilla coelebs*)[140], a diurnal migrant, red-winged blackbirds from all treatment groups failed to exhibit any migratory activity or orientation behaviour in the Emlen funnels. While it is possible that red-winged blackbirds may show orientation behaviour in funnels when tested outside under clear skies as opposed to in captivity, recommendations for future research would be to solely utilize European starlings, which acclimate quickly to handling, transportation, and placement into funnels.

This study also indicated the suitability of both the digital video-tracking system for simultaneous analysis of multiple birds, as well as the novel analysis of bird movements within the funnels using BirdOriTrack[71] software. The video-tracking program combined with our modified Emlen funnels would be highly suitable for future research because it is inexpensive, can be used either in the field or a laboratory setting, and provides sensitive and accurate data on the birds' movements. The use of BirdOriTrack for analysis of behavioural data is free, user-

friendly, and provides an objective and precise analysis of migratory activity and orientation. Eliminating the use of thermal paper and microswitches will be beneficial for future research concerning motion data, as it could promote data sharing between researchers, allow for post-hoc analyses[71], and increases the validity and robustness of experiments examining migratory behaviour.

4.2 Important findings on developmental and behavioural changes resulting from early exposure to Aroclor 1254 in a passerine species

Initial findings from our pilot study indicated that exposure of nestling European starlings and red-winged blackbirds to environmentally relevant levels of an endocrine disrupting mixture, Aroclor 1254, did not result in significant alterations in survival, nor were there any major short-term effects on growth parameters. While birds from all treatment groups did exhibit a significant increase in wing chord fluctuating asymmetry (FA) when compared to controls, these differences did not persist past the period of nestling development. Though minor when compared to changes in wing chord FA, tarsus length FA in birds from low and high treatment groups also showed a significant increase when compared to controls, further supporting the existence of relatively subtle short-term effects during the nestling exposure. Therefore, it is important to further examine the potential for any latent effects that could be more relevant for individual fitness.

Findings from Chapter 3 indicate that there is a link between early exposure to EDCs and latent alterations in migratory behaviour. While all captive birds were successfully brought into autumn migratory condition, PCB-exposed European starlings were significantly delayed in their ability to correctly orient. High-dose males were also significantly delayed in their completion of

primary moult when compared to untreated males. Although no causal mechanism was examined, PCB congeners can interact with the estrogen and androgen receptors[25] and so there could have been some level of disruption of sex steroid hormone homeostasis. This could be a potential mechanism contributing to the sex-related differences in moult completion seen in our study; however, additional research is required. While there will always be inherently complex and variable toxic responses of avian species to PCBs, it is necessary to make associations between tissue burdens of PCBs to field observations of toxicity[141]. In previous studies of European starlings residing in a heavily contaminated Superfund site, there were observed reductions in nestling body weight and fledging success, as well as higher nestling mortality in second broods. In addition to these significant alterations in nest productivity, there was reduced nest site attentiveness exhibited by adult starlings. These effects were associated with 52.5 μg/g and 15.9 μg/g ∑Aroclor 1254 in chick and adult whole body homogenates, respectively. This range of concentrations associated with behavioural effects found in adult starlings is comparable to our study, in which levels of Aroclor 1254 (μg/g tissue) quantified in liver tissues of birds ranged from 8.01 μg/g to 38.61 μg/g. Also, adult ring doves continuously exposed to dietary 10 ppm Aroclor 1254, exhibited reduced parental attentiveness, which was associated with 15 ppm Aroclor 1254 in the liver tissue. These results of behavioural impairments, combined with the lack of acute effects on nestling success or productivity, further support the significance of our observed latent behavioural effects on migratory orientation. Tissue PCB residues associated with effects on early development of altricial wild birds generally range from 0.20 μg/g to 7.7 μg/g wet weight[141], levels which are mostly above the Aroclor 1254 residues quantified in liver tissues from the present study.

The study area (Saskatchewan) from which our experimental animals were taken represents one of the least PCB-contaminated regions of Canada[16]. Thus dosage levels from our study do not reflect what birds are being exposed to in this region. However, studies on migratory birds within contaminated regions, such as the North American Great Lakes region, have reported PCB levels ranging from > 5 ng/L to 500 ng/L in rivers and estuaries[16]. Quantification of total estrogen equivalents in various soil, sediment, or invertebrate prey from a range of areas to compare to our dose levels would be useful for extrapolating the magnitude of effects that might be seen in the field based on these low-level experimental exposures. Studies in which birds are exposed to low or background levels of PCBs or other endocrine disrupting compounds may reveal only subtle or no acute effects but may still be important for migration.

To the best of our knowledge, this is only the third study in which migratory behaviour has been examined in a passerine songbird after exposure to an environmental contaminant. Adult European robins (*Erithacus rubecula*), a nocturnal migrant, were exposed to 11-13 mealworms injected with low levels of Clophen A50 (a PCB) over a period of two days, which resulted in significantly higher average migratory activity than that of control birds[142]. Migratory restlessness is a critical component of the suite of events preceding a migratory event; however, that study did not examine migratory orientation. Over 20 years later, another study supplemented these findings by demonstrating that migratory orientation could be altered in adult white-throated sparrows (*Zonotrichia albicollis*) upon exposure to acephate, a common organophosphorus neurotoxic pesticide[143]. Control and exposed juveniles moved in a preferred and seasonally correct direction when placed in funnels during a fall migration; however, adults displayed random activity, suggesting that there was some level of interference with neurological

function associated with memory of prior migration route and wintering grounds rather than innate compass orientation.

4.3 Implications for future research

Further research should aim to utilize both juveniles and adults during an autumn migration, to allow for a comparison of migratory activity and orientation ability. Since juveniles and adults utilize different migration strategies, it is important to further elucidate if exposure to a PCB mixture could interfere with learning and memory of a migratory route in adult birds. In a follow-up learning study of the same birds used in this study, Zahara *et al*. (in prep) found that starlings in the high dose group failed to learn a spatial task. This in combination with our results demonstrates that there is likely some level of PCB-induced interference with the innate mechanism of orientation employed by juvenile birds. Examination of any differences in hippocampal volume and total neuron numbers between exposed migratory juveniles and adults will be beneficial for understanding the potential impact of hormone-induced alterations on hippocampal structure. The hippocampus has been shown to be essential in spatial processing, learning and memory[128, 129, 144] and in the overall regulation of navigational behaviour[145]. It expresses receptors for thyroid hormones, gonadal steroids, glucocorticoids, and mineralocorticoids, and predictably, has been proven to be sensitive to changes in circulating levels of hormones[132, 133]. Food-storing birds that rely largely upon the processing of spatial information for locating caches of food have been shown to have enlarged hippocampi[128, 129, 144] and so it would be interesting to determine if these hippocampal characteristics were also present in migratory species.

This study also opens up many possibilities for studying other individual chemicals or mixtures that may similarly interfere with various components of memory and endogenous programs of orientation. It is far more relevant to examine adverse outcome pathways resulting from exposure to mixtures of EDCs, since they exist in the environment as complex mixtures of individual congeners[9]. While difficult, making extrapolations of the molecular, biochemical, and cellular level effects of EDCs to adverse outcomes on whole individuals and populations is far more relevant when conducting risk assessments of avian wildlife populations and implementing conservation strategies[1, 85, 107]. The significance of these higher order effects is not well understood and is currently lacking in risk assessments of avian wildlife populations. Migratory activity and orientation behaviour using the methods described here could be adopted as a sensitive reproducible biomarker for measuring the effects of chemical stressors during different life stages.

Data from this study suggests that developmental exposure to Aroclor 1254, a developmental neurotoxicant, resulted in altered navigation and orientation abilities of European starlings. These findings indicate that exposure to environmentally relevant concentrations of known neurotoxicants could cause subtle long-term effects on neuroendocrine components relating to behaviour, potentially resulting in deleterious effects on fitness and survival[53].

5 REFERENCES

1. Ottinger, M.A.; Carro, T.; Bohannon, M.; Baltos, L.; Marcell, A.M.; McKernan, M.; Dean, K.M.; Lavoie, E.; Abdelnabi, M. Assessing effects of environmental chemicals on neuroendocrine systems: potential mechanisms and functional outcomes. *Gen. Comp. Endocrinol.* **2013,** *190,* 194-202; 10.1016/j.ygcen.2013.06.004.

2. Beyer, A. and Biziuk, M. Environmental fate and global distribution of polychlorinated biphenyls. *Rev. Environ. Contam. Toxicol.* **2009,** *201,* 137-158; 10.1007/978-1-4419-0032-6_5.

3. Diamanti-Kandarakis, E.; Bourguignon, J.P.; Giudice, L.C.; Hauser, R.; Prins, G.S.; Soto, A.M.; Zoeller, R.T.; Gore, A.C. Endocrine-disrupting chemicals: an Endocrine Society scientific statement. *Endocr. Rev.* **2009,** *30* (4), 293-342; 10.1210/er.2009-0002.

4. Corsolini, S. Non-Pesticide Endocrine Disrupters And Reproductive Health. In Reproductive Health and the Environment. Series: Environmental Science and Technology Library, Vol. 22. Nicolopoulou-Stamati, P., Hens, L. and Howard, C.V. Eds.; Springer, Dordrecht (NL), **2007**; pp. 161-186.

5. Zoeller, R.T.; Brown, T.R.; Doan, L.L.; Gore, A.C.; Skakkebaek, N.E.; Soto, A.M.; Woodruff, T.J.; Vom Saal, F.S. Endocrine-disrupting chemicals and public health protection: a statement of principles from The Endocrine Society. *Endocrinology* **2012,** *153* (9), 4097-4110; 10.1210/en.2012-1422.

6. Poland, A. and Knutson, J.C. 2,3,7,8-Tetrachlorodibenzo-P-Dioxin and Related Halogenated Aromatic Hydrocarbons: Examination of the Mechanism of Toxicity. *Annu. Rev. Pharmacol. Toxicol.* **1982,** *22,* 517-554; 10.1146/annurev.pa.22.040182.002505.

7. Poland, A.; Glover, E.; Kende, A.S. Stereospecific, high affinity binding of 2,3,7,8-tetrachlorodibenzo-p-dioxin by hepatic cytosol. Evidence that the binding species is receptor for induction of aryl hydrocarbon hydroxylase. *J. Biol. Chem.* **1976,** *251* (16), 4936-4946.

8. Head, J.A.; Hahn, M.E.; Kennedy, S.W. Key amino acids in the aryl hydrocarbon receptor predict dioxin sensitivity in avian species. *Environ. Sci. Technol.* **2008,** *42* (19), 7535-7541.

9. Erickson, M.D. and Kaley, R.G.I. Applications of polychlorinated biphenyls. *Environ. Sci. Pollut. Res. Int.* **2011,** *18* (2), 135-151; 10.1007/s11356-010-0392-1.

10. Kafafi, S.A.; Afeefy, H.Y.; Ali, A.H.; Said, H.K.; Kafafi, A.G. Binding of polychlorinated biphenyls to the aryl hydrocarbon receptor. *Environ. Health Perspect.* **1993,** *101* (5), 422-428.

11. Ahlborg, U.; Becking, G.; Birnbaum, L.; Brouwer, A.; Derks, H.; Feeley, M.; Golor, G.; Hanberg, A.; Larsen, J.; Liem, A.; Safe, S.; Schlatter, C.; Waern, F.; Younes, M.; Yrjänheikki, E. Toxic equivalency factors for dioxin-like PCBs: Report on WHO-ECEH and IPCS consultation, December 1993. *Chemosphere* **1994,** *28* (6), 1049-1067; 10.1016/0045-6535(94)90324-7.

12. Hahn, M.E. Aryl hydrocarbon receptors: diversity and evolution. *Chem. Biol. Interact.* **2002,** *141* (1-2), 131-160; 10.1016/S0009-2797(02)00070-4.

13. Jobling, S. and Tyler, C.R. Introduction: The ecological relevance of chemically induced endocrine disruption in wildlife. *Environ. Health Perspect.* **2006,** *114 Suppl 1*, 7-8.

14. Breivik, K.; Sweetman, A.; Pacyna, J.M.; Jones, K.C. Towards a global historical emission inventory for selected PCB congeners--a mass balance approach 3. An update. *Sci. Total Environ.* **2007,** *377* (2-3), 296-307; 10.1016/j.scitotenv.2007.02.026.

15. Safe, S.H. Polychlorinated biphenyls (PCBs): environmental impact, biochemical and toxic responses, and implications for risk assessment. *Crit. Rev. Toxicol.* **1994,** *24* (2), 87-149; 10.3109/10408449409049308.

16. Jaspers, V.L.B.; Dirtu, A.C.; Eens, M.; Neels, H.; Covaci, A. Predatory Bird Species Show Different Patterns of Hydroxylated Polychlorinated Biphenyls (HO-PCBs) and Polychlorinated Biphenyls (PCBs). *Environ. Sci. Technol.* **2008,** *42* (9), 3465-3471; 10.1021/es7030455.

17. Kalmaz, E.V. and Kalmaz, G.D. Transport, distribution and toxic effects of polychlorinated biphenyls in ecosystems: Review. *Ecol. Model.* **1979,** *6* (3), 223-251; 10.1016/0304-3800(79)90014-0.

18. Environment Canada Canadian soil Quality Guidelines for Polychlorinated Biphenyls (PCBs): Environmental Health. Scientific Supporting Document. Ecosystem Health: Science-based Solutions Report No. 1-2. National Guidelines and Standards Office, Environmental Quality Branch, Environment Canada. Ottawa. **2001,** 1-107.

19. Henshel, D.S. Developmental neurotoxic effects of dioxin and dioxin-like compounds on domestic and wild avian species. *Environ. Toxicol. Chem.* **1998,** *17*, 88-98; 10.1002/etc.5620170111.

20. McFarland, V.A. and Clarke, J.U. Environmental Occurrence, Abundance, and Potential Toxicity of Polychlorinated Biphenyl Congeners: Considerations for a Congener-Specific Analysis. *Environ. Health Perspect.* **1989,** *81*, 225-239.

21. Morrissey, C.A.; Stanton, D.W.; Pereira, M.G.; Newton, J.; Durance, I.; Tyler, C.R.; Ormerod, S.J. Eurasian dipper eggs indicate elevated organohalogenated contaminants in urban rivers. *Environ. Sci. Technol.* **2013,** *47* (15), 8931-8939; 10.1021/es402124z.

22. Arenal, C.A.; Halbrook, R.S.; Woodruff, M. European starling (*Sturnus vulgaris*): avian model and monitor of polychlorinated biphenyl contamination at a Superfund site in southern Illinois, USA. *Environ. Toxicol. Chem.* **2004,** *23* (1), 93-104; 10.1897/02-606.

23. Safe, S. and Hutzinger, O. Polychlorinated Biphenyls (PCBs) and Polybrominated Biphenyls (PBBs): Biochemistry, Toxicology, and Mechanism of Action. *Crit. Rev. Toxicol.* **1984,** *13* (4), 319-395; 10.3109/10408448409023762.

24. Anonymous Agency for Toxic Substances and Disease Registry (ATSDR). 2000. Toxicological profile for Polychlorinated Biphenyls (PCBs). Atlanta, GA: U.S. Department of Health and Human Services, Public Health Service.

25. Sonnenschein, C. and Soto, A.M. An updated review of environmental estrogen and androgen mimics and antagonists. *J. Steroid Biochem. Mol. Biol.* **1998,** *65* (1-6), 143-150.

26. Bonefeld-Jorgensen, E.C.; Andersen, H.R.; Rasmussen, T.H.; Vinggaard, A.M. Effect of highly bioaccumulated polychlorinated biphenyl congeners on estrogen and androgen receptor activity. *Toxicology* **2001,** *158* (3), 141-153.

27. Takeuchi, S.; Shiraishi, F.; Kitamura, S.; Kuroki, H.; Jin, K.; Kojima, H. Characterization of steroid hormone receptor activities in 100 hydroxylated polychlorinated biphenyls, including congeners identified in humans. *Toxicology* **2011,** *289* (2-3), 112-121; 10.1016/j.tox.2011.08.001.

28. Doering, J.A.; Wiseman, S.; Beitel, S.C.; Giesy, J.P.; Hecker, M. Identification and expression of aryl hydrocarbon receptors (AhR1 and AhR2) provide insight in an evolutionary context regarding sensitivity of white sturgeon (*Acipenser transmontanus*) to dioxin-like compounds. *Aquatic Toxicology* **2014,** *150* (0), 27-35; http://dx.doi.org.cyber.usask.ca/10.1016/j.aquatox.2014.02.009.

29. Kawajiri, K. and Fujii-Kuriyama, Y. Cytochrome P450 gene regulation and physiological functions mediated by the aryl hydrocarbon receptor. *Arch. Biochem. Biophys.* **2007,** *464* (2), 207-212; 10.1016/j.abb.2007.03.038.

30. Fonnum, F. and Mariussen, E. Mechanisms involved in the neurotoxic effects of environmental toxicants such as polychlorinated biphenyls and brominated flame retardants. *J. Neurochem.* **2009,** *111* (6), 1327-1347; 10.1111/j.1471-4159.2009.06427.x.

31. Brouwer, A.; Morse, D.C.; Lans, M.C.; Schuur, A.G.; Murk, A.J.; Klasson-Wehler, E.; Bergman, A.; Visser, T.J. Interactions of Persistent Environmental Organohalogens With the Thyroid Hormone System: Mechanisms and Possible Consequences for Animal and Human Health. *Toxicol. Ind. Health* **1998,** *14* (1-2), 59-84; 10.1177/074823379801400107.

32. Park, J.S.; Holden, Y.; Chang, S.; Heckly, S.; McKeown, K.; Jewell, N.; Hooper, K. Hydroxylated metabolites of polychlorinated biphenyls (PCBs) in California wild birds. *Organohalogen Compd* **2008,** *70*, 157-160.

33. Markman, S.; Leitner, S.; Catchpole, C.; Barnsley, S.; Muller, C.T.; Pascoe, D.; Buchanan, K.L. Pollutants increase song complexity and the volume of the brain area HVC in a songbird. *PLoS One* **2008,** *3* (2), e1674; 10.1371/journal.pone.0001674.

34. Markman, S.; Müller, C.T.; Pascoe, D.; Dawson, A.; Buchanan, K.L. Pollutants affect development in nestling starlings *Sturnus vulgaris*. *J. Appl. Ecol.* **2011,** *48*, 391-397; 10.1111/j.1365-2664.2010.01931.x.

35. McNabb, F.M.A. and Fox, G.A. Avian thyroid development in chemically contaminated environments: is there evidence of alterations in thyroid function and development? *Evol. Dev.* **2003,** *5*, 76-82; 10.1046/j.1525-142X.2003.03012.x.

36. Moccia, R.D.; Fox, G.A.; Britton, A. A quantitative assessment of thyroid histopathology of herring gulls (*Larus argentatus*) from the Great Lakes and a hypothesis on the causal role of environmental contaminants. *J. Wildl. Dis.* **1986,** *22* (1), 60-70.

37. Darras, V.M. Endocrine disrupting polyhalogenated organic pollutants interfere with thyroid hormone signalling in the developing brain. *Cerebellum* **2008,** *7* (1), 26-37; 10.1007/s12311-008-0004-5.

38. Peakall, D.B. Disrupted patterns of behavior in natural populations as an index of ecotoxicity. *Environ. Health Perspect.* **1996,** *104 Suppl 2*, 331-335.

39. Hoogesteijn, A.L.; Kollias, G.V.; Quimby, F.W.; De Caprio, A.P.; Winkler, D.W.; DeVoogd, T.J. Development of a brain nucleus involved in song production in zebra finches (*Taeniopygia guttata*) is disrupted by Aroclor 1248. *Environ. Toxicol. Chem.* **2008,** *27*, 2071-2075; 10.1897/07-484.1.

40. Fry, D.M. Reproductive effects in birds exposed to pesticides and industrial chemicals. *Environ. Health Perspect.* **1995,** *103 Suppl 7*, 165-171.

41. Porterfield, S.P. Thyroidal dysfunction and environmental chemicals--potential impact on brain development. *Environ. Health Perspect.* **2000,** *108 Suppl 3*, 433-438.

42. Yasui, T.; Kim, E.; Iwata, H.; Tanabe, S. Identification of aryl hydrocarbon receptor 2 in aquatic birds; cDNA cloning of AHR1 and AHR2 and characteristics of their amino acid sequences. *Mar. Environ. Res.* **2004,** *58* (2-5), 113-118; 10.1016/j.marenvres.2004.03.004.

43. Farmahin, R.; Manning, G.E.; Crump, D.; Wu, D.; Mundy, L.J.; Jones, S.P.; Hahn, M.E.; Karchner, S.I.; Giesy, J.P.; Bursian, S.J.; Zwiernik, M.J.; Fredricks, T.B.; Kennedy, S.W. Amino acid sequence of the ligand-binding domain of the aryl hydrocarbon receptor 1 predicts sensitivity of wild birds to effects of dioxin-like compounds. *Toxicol. Sci.* **2013,** *131* (1), 139-152; 10.1093/toxsci/kfs259.

44. Whitlock, J.P. INDUCTION OF CYTOCHROME P4501A1. *Annu. Rev. Pharmacol. Toxicol.* **1999,** *39* (1), 103-125; 10.1146/annurev.pharmtox.39.1.103.

45. Visser, T.J.; Kaptein, E.; Gijzel, A.L.; de Herder, W.W.; Ebner, T.; Burchell, B. Glucuronidation of thyroid hormone by human bilirubin and phenol UDP-glucuronyltransferase isoenzymes. *FEBS Lett.* **1993,** *324* (3), 358-360; 10.1016/0014-5793(93)80151-J.

46. Van Birgelen, A.P.; Smit, E.A.; Kampen, I.M.; Groeneveld, C.N.; Fase, K.M.; Van der Kolk, J.; Poiger, H.; Van den Berg, M.; Koeman, J.H.; Brouwer, A. Subchronic effects of 2,3,7,8-

TCDD or PCBs on thyroid hormone metabolism: use in risk assessment. *Eur. J. Pharmacol.* **1995,** *293* (1), 77-85.

47. Merryman, J.I. and Buckles, E.L. The Avian Thyroid Gland. Part Two: A Review of Function and Pathophysiology. *J. Avian Med. Surg.* **1998,** *12* (4), pp. 238-242.

48. Nair, G.A.; Pant, K.; Chandola-Saklani, A. Environmental and hormonal control of vernal migration in redheaded bunting (*Emberiza bruniceps*). *J. Biosci.* **1994,** *19*, 453-466; 10.1007/BF02703181.

49. Weiss, B. Endocrine disruptors as a threat to neurological function. *J. Neurol. Sci.* **2011,** *305*, 11-21; 10.1016/j.jns.2011.03.014.

50. McNabb, F.M. Avian thyroid development and adaptive plasticity. *Gen. Comp. Endocrinol.* **2006,** *147* (2), 93-101; 10.1016/j.ygcen.2005.12.011.

51. Olson, J.M.; McNabb, F.M.; Jablonski, M.S.; Ferris, D.V. Thyroid development in relation to the development of endothermy in the red-winged blackbird (*Agelaius phoeniceus*). *Gen. Comp. Endocrinol.* **1999,** *116* (2), 204-212; 10.1006/gcen.1999.7363.

52. Výboh, P.; Zeman, M.; Juráni, M.; Buyse, J.; Decuypere, E. Plasma thyroid hormone and growth hormone patterns in precocial Japanese quail and altricial European starlings during postnatal development. *Comp. Biochem. Physiol. C Pharmacol. Toxicol. Endocrinol.* **1996,** *114* (1), 23-27; 10.1016/0742-8413(95)02106-X.

53. Ottinger, M.A.; Lavoie, E.; Thompson, N.; Barton, A.; Whitehouse, K.; Barton, M.; Abdelnabi, M.; Quinn, M.,Jr; Panzica, G.; Viglietti-Panzica, C. Neuroendocrine and behavioral effects of embryonic exposure to endocrine disrupting chemicals in birds. *Brain Res. Rev.* **2008,** *57* (2), 376-385; 10.1016/j.brainresrev.2007.08.011.

54. Jenssen, B.M.; Aarnes, J.B.; Murvoll, K.M.; Herzke, D.; Nygard, T. Fluctuating wing asymmetry and hepatic concentrations of persistent organic pollutants are associated in European shag (*Phalacrocorax aristotelis*) chicks. *Sci. Total Environ.* **2010,** *408* (3), 578-585; 10.1016/j.scitotenv.2009.10.036.

55. Ottinger, M.A.; Lavoie, E.T.; Abdelnabi, M.; Quinn, M.J.,Jr; Marcell, A.; Dean, K. An overview of dioxin-like compounds, PCB, and pesticide exposures associated with sexual differentiation of neuroendocrine systems, fluctuating asymmetry, and behavioral effects in birds. *J. Environ. Sci. Health. C. Environ. Carcinog. Ecotoxicol. Rev.* **2009,** *27* (4), 286-300; 10.1080/10590500903310229.

56. Ricklefs, R.E. Patterns of growth in birds. V. A comparative study of the growth and development in the starling, common tern, and Japanese quail. *Auk* **1979,** *96*, 10-30.

57. Choi, I.H.; Ricklefs, R.E.; Shea, R.E. Skeletal Muscle Growth, Enzyme Activities, and the Development of Thermogenesis: A Comparison between Altricial and Precocial Birds. *Physiol. Zool.* **1993,** *66*, 455-473.

58. Masters Vleck, C. and Vleck, D. Patterns of Metabolism and Growth in Avian Embryos. *Integr. Comp. Biol.* **1980,** *20*, 405-416; 10.1093/icb/20.2.405.

59. Kumar, V.; Wingfield, J.C.; Dawson, A.; Ramenofsky, M.; Rani, S.; Bartell, P. Biological clocks and regulation of seasonal reproduction and migration in birds. *Physiol. Biochem. Zool.* **2010,** *83* (5), 827-835; 10.1086/652243.

60. Dawson, A. Control of the annual cycle in birds: endocrine constraints and plasticity in response to ecological variability. *Philos. Trans. R. Soc. Lond. B. Biol. Sci.* **2008,** *363* (1497), 1621-1633; 10.1098/rstb.2007.0004.

61. Gwinner, E. Circannual clocks in avian reproduction and migration. *Ibis* **1996,** *138*, 47-63; 10.1111/j.1474-919X.1996.tb04312.x.

62. Dawson, A. Plasma-Luteinizing Hormone and Prolactin During Circannual Rhythms of Gonadal Maturation and Molt in Male and Female European Starlings. *J. Biol. Rhythms* **1997,** *12* (4), 371-377; 10.1177/074873049701200409.

63. Wingfield, J.C. Flexibility in annual cycles of birds: implications for endocrine control mechanisms. *Journal of Ornithology* **2005,** *146*, 291-304.

64. Wingfield, J.C. Comparative endocrinology, environment and global change. *Gen. Comp. Endocrinol.* **2008,** *157* (3), 207-216; 10.1016/j.ygcen.2008.04.017.

65. Deviche, P. Androgen Regulation of Avian Premigratory Hyperphagia and Fattening: From Eco-Physiology to Neuroendocrinology. *Integr. Comp. Biol.* **1995,** *35*, 234-245; 10.1093/icb/35.3.234.

66. Ramenofsky, M. and Wingfield, J.C. Regulation of Migration. *BioScience* **2007,** *57 (2)*, 135-143; 10.1641/B570208.

67. Landys-Ciannelli, M.M.; Ramenofsky, M.; Piersma, T.; Jukema, J.; Wingfield, J.C.; Castricum Ringing Group. Baseline and stress-induced plasma corticosterone during long-distance migration in the bar-tailed godwit, *Limosa lapponica*. *Physiol. Biochem. Zool.* **2002,** *75* (1), 101-110; 10.1086/338285.

68. Schaub, M. and Jenni, L. Variation of Fuelling Rates among Sites, Days and Individuals in Migrating Passerine Birds. *Funct. Ecol.* **2001,** *15 (5)*, 584-594.

69. Emlen, S.T. and Emlen, J.T. A Technique for Recording Migratory Orientation of Captive Birds. *Auk* **1966,** *83* (3), 361-367.

70. Pathak, V.K. and Chandola, A. Involvement of thyroid gland in the development of migratory disposition in the redheaded bunting, *Emberiza bruniceps*. *Horm. Behav.* **1982,** *16* (1), 46-58.

71. McNabb, F.M.A. The Hypothalamic-Pituitary-Thyroid (HPT) Axis in Birds and Its Role in Bird Development and Reproduction. *Crit. Rev. Toxicol.* **2007,** *37* (1-2), 163-193; 10.1080/10408440601123552.

72. Muheim, R.; Henshaw, I.; Sjöberg, S.; Deutschlander, M.E. BirdOriTrack: a new video-tracking program for orientation research with migratory birds. *J. Field Ornithol.* **2014,** *85*, 91-105; 10.1111/jofo.12053.

73. Mouritsen, H. and Larsen, O.N. Migrating songbirds tested in computer-controlled Emlen funnels use stellar cues for a time-independent compass. *J. Exp. Biol.* **2001,** *204* (Pt 22), 3855-3865.

74. Government of Canada. (2010c). Migratory Birds Convention Act (S.C. 1994, c. 22). Department of Justice. Retrieved from: http://laws-lois.justice.gc.ca/eng/acts/M-7.01/20030101/P1TT3xt3.html.

75. Linz, G.M.; Homan, J.H.; Gaulker, S.M.; Penry, L.B.; Bleier, W.J. European starlings: a review of an invasive species with far-reaching impacts. In Managing invasive vertebrate species: proceedings of an international symposium. USDA/APHIS/National Wildlife Research Center, Fort Collins, Colorado, USA. G. W. Witmer, W. C. Pitt, and K. A. Fagerstone, Eds.; **2007**; pp. 378-386.

76. Kessel, B. Distribution and Migration of the European Starling in North America. *Condor* **1953,** *55*, 49-67.

77. Berthold, P. Genetic control of migratory behaviour in birds. *Trends Ecol. Evol.* **1991,** *6* (8), 254-257; 10.1016/0169-5347(91)90072-6.

78. Homan, H.; Linz, G.; Engeman, R.; Penry, L. Spring dispersal patterns of red-winged blackbirds, *Agelaius phoeniceus*, staging in eastern South Dakota. *Can. Field-Nat.* **2004,** *118* (2), 201-209.

79. Holcomb, L.C. Prolonged Incubation Behaviour of Red-Winged Blackbird Incubating several Egg Sizes. *Behaviour* **1970,** *36*, 74-83; 10.1163/156853970X00051.

80. Bateson, M. and Feenders, G. The use of passerine bird species in laboratory research: implications of basic biology for husbandry and welfare. *ILAR J.* **2010,** *51* (4), 394-408; 10.1093/ilar.51.4.394.

81. Dolbeer, R. Movement and Migration Patterns of Red-Winged Blackbirds - Continental Overview. *Bird-Banding* **1978,** *49* (1), 17-34.

82. Cheek, A.O. Subtle sabotage: endocrine disruption in wild populations. *Rev. Biol. Trop.* **2006,** *54*, 1-19.

83. Panzica, G.C.; Viglietti-Panzica, C.; Mura, E.; Quinn, M.J.,Jr; Lavoie, E.; Palanza, P.; Ottinger, M.A. Effects of xenoestrogens on the differentiation of behaviorally-relevant neural circuits. *Front. Neuroendocrinol.* **2007,** *28* (4), 179-200; 10.1016/j.yfrne.2007.07.001.

84. Kavlock, R.; Chandler, K.; Houck, K.; Hunter, S.; Judson, R.; Kleinstreuer, N.; Knudsen, T.; Martin, M.; Padilla, S.; Reif, D.; Richard, A.; Rotroff, D.; Sipes, N.; Dix, D. Update on EPA's ToxCast program: providing high throughput decision support tools for chemical risk management. *Chem. Res. Toxicol.* **2012,** *25* (7), 1287-1302; 10.1021/tx3000939.

85. Colborn, T.; vom Saal, F.S.; Soto, A.M. Developmental effects of endocrine-disrupting chemicals in wildlife and humans. *Environ. Health Perspect.* **1993,** *101* (5), 378-384.

86. Kavlock, R.J.; Daston, G.P.; DeRosa, C.; Fenner-Crisp, P.; Gray, L.E.; Kaattari, S.; Lucier, G.; Luster, M.; Mac, M.J.; Maczka, C.; Miller, R.; Moore, J.; Rolland, R.; Scott, G.; Sheehan, D.M.; Sinks, T.; Tilson, H.A. Research needs for the risk assessment of health and environmental effects of endocrine disruptors: a report of the U.S. EPA-sponsored workshop. *Environ. Health Perspect.* **1996,** *104 Suppl 4*, 715-740; 1289/ehp.96104s4715.

87. Houde, M.; Pacepavicius, G.; Wells, R.S.; Fair, P.A.; Letcher, R.J.; Alaee, M.; Bossart, G.D.; Hohn, A.A.; Sweeney, J.; Solomon, K.R.; Muir, D.C. Polychlorinated biphenyls and hydroxylated polychlorinated biphenyls in plasma of bottlenose dolphins (*Tursiops truncatus*) from the Western Atlantic and the Gulf of Mexico. *Environ. Sci. Technol.* **2006,** *40* (19), 5860-5866; 10.1021/es060629n.

88. Klasson-Wehler, E.; Bergman, A.; Athanasiadou, M.; Ludwig, J.P.; Auman, H.J.; Kannan, K.; Van Den Berg, M.; Murk, A.J.; Feyk, L.A.; Giesy, J.P. Hydroxylated and methylsulfonyl polychlorinated biphenyl metabolites in albatrosses from Midway Atoll, North Pacific Ocean. *Environ. Toxicol. Chem.* **1998,** *17* (8), 1620-1625.

89. Kunisue, T. and Tanabe, S. Hydroxylated polychlorinated biphenyls (OH-PCBs) in the blood of mammals and birds from Japan: Lower chlorinated OH-PCBs and profiles. *Chemosphere* **2009,** *74* (7), 950-961; 10.1016/j.chemosphere.2008.10.038.

90. McKinney, J.D. and Waller, C.L. Polychlorinated biphenyls as hormonally active structural analogues. *Environ. Health Perspect.* **1994,** *102* (3), 290-297.

91. Gopukumar, N.N.; Kanchan, P.; Chandola-Saklani, A. Environmental and hormonal control of vernal migration in redheaded bunting (*Emberiza bruniceps*). *J. Biosci.* **1994,** *19*, 453-466; 10.1007/BF02703181.

92. Heinz, G.H.; Hill, E.F.; Contrera, J.F. Dopamine and Norepinephrine Depletion in Ring Doves Fed DDE, Dieldrin, and Aroclor 1254. *Toxicol. Appl. Pharmacol.* **1980,** *53* (1), 75-82; 10.1016/0041-008X(80)90383-X.

93. Pinheiro, J.; Bates, D.; DebRoy, S.; Sarkar, D.; R Core Team. *nlme: Linear and Nonlinear Mixed Effects Models. R package version 3.1-107. 4.2. See* http://cran.r-project.org/web/packages/nlme/index.html. **2013,** .

94. Burnham, K.P. and Anderson, D.R. *Model selection and multimodal inference: a practical information-theoretic approach.* Springer-Verlag: New York, USA, 2002; pp. 488.

95. Rosario-Martinez, H.D. *phia: post-hoc interaction analysis. R package version 0.1–3.* See http://CRAN.R-project.org/package= phia. **2013**.

96. Love, O.P.; Shutt, L.J.; Silfies, J.S.; Bortolotti, G.R.; Smits, J.E.; Bird, D.M. Effects of dietary PCB exposure on adrenocortical function in captive American kestrels (*Falco sparverius*). *Ecotoxicology* **2003,** *12* (1-4), 199-208.

97. Fernie, K.; Bortolotti, G.; Smits, J. Reproductive abnormalities, teratogenicity, and developmental problems in American kestrels (*Falco sparverius*) exposed to polychlorinated biphenyls. *J. Toxicol. Environ. Health A* **2003,** *66* (22), 2089-2103; 10.1080/15287390390211270.

98. Hoogesteijn, A.; DeVoogd, T.; Quimby, F.; De Caprio, T.; Kollias, G. Reproductive impairment in zebra finches (*Taeniopygia guttata*). *Environ. Toxicol. Chem.* **2005,** *24* (1), 219-223; 10.1897/03-632R.1.

99. Schew, W.A.; McNabb, F.M.; Scanes, C.G. Comparison of the ontogenesis of thyroid hormones, growth hormone, and insulin-like growth factor-I in ad libitum and food-restricted (altricial) European starlings and (precocial) Japanese quail. *Gen. Comp. Endocrinol.* **1996,** *101* (3), 304-316; 10.1006/gcen.1996.0033.

100. Graham, J.H.; Emlen, J.M.; Freeman, D.C. Developmental stability and its applications in ecotoxicology. *Ecotoxicology* **1993,** *2* (3), 175-184; 10.1007/BF00116422.

101. Palmer, A.R. and Strobeck, C. Fluctuating Asymmetry: Measurement, Analysis, Patterns. *Annu. Rev. Ecol. Syst.* **1986,** *17* (- 1), 391-421; 10.1146/annurev.es.17.110186.002135.

102. Clarke, G.M. Fluctuating asymmetry of invertebrate populations as a biological indicator of environmental quality. *Environ. Pollut.* **1993,** *82* (2), 207-211.

103. Bustnes, J.O.; Folstad, I.; Erikstad, K.E.; Fjeld, M.; Miland, ØO.; Skaare, J. Blood concentration of organochlorine pollutants and wing feather asymmetry in Glaucous Gulls. *Funct. Ecol.* **2002,** *16* (5), 617-622; doi: 10.1046/j.1365-2435.2002.00656.x.

104. Eeva, T.; Tanhuanpaa, S.; Rabergh, C.; Airaksinen, S.; Nikinmaa, M.; Lehikoinen, E. Biomarkers and fluctuating asymmetry as indicators of pollution-induced stress in two hole-nesting passerines. *Funct. Ecol.* **2000,** *14*, 235-243.

105. Beasley, D.A.E.; Bonisoli-Alquati, A.; Mousseau, T.A. The use of fluctuating asymmetry as a measure of environmentally induced developmental instability: A meta-analysis. *Ecol. Ind.* **2013,** *30* (0), 218-226; http://dx.doi.org/10.1016/j.ecolind.2013.02.024.

106. Thomas, A.L.R. The Aerodynamic Costs of Asymmetry in the Wings and Tail of Birds: Asymmetric Birds can't Fly round Tight Corners. *Proc. R. Soc. Lond. B* **1993,** *254*, 181-189; 10.1098/rspb.1993.0144.

107. Mauck, W.L.; Mehrle, P.M.; Mayer, F.L. Effects of the Polychlorinated Biphenyl Aroclor® 1254 on Growth, Survival, and Bone Development in Brook Trout (*Salvelinus fontinalis). J. Fish. Res. Board Can* **1978,** *35*, 1084-1088; 10.1139/f78-171.

108. Andrews, J.E. Polychlorinated biphenyl (Aroclor 1254) induced changes in femur morphometry calcium metabolism and nephrotoxicity. *Toxicology* **1989,** *57* (1), 83-96.

109. Hooper, M.J.; Ankley, G.T.; Cristol, D.A.; Maryoung, L.A.; Noyes, P.D.; Pinkerton, K.E. Interactions between chemical and climate stressors: a role for mechanistic toxicology in assessing climate change risks. *Environ. Toxicol. Chem.* **2013,** *32* (1), 32-48; 10.1002/etc.2043.

110. Fox, G.A.; Gilman, A.P.; Peakall, D.B.; Anderka, F.W. Behavioral Abnormalities of Nesting Lake Ontario Herring Gulls. *J. Wildl. Manage.* **1978,** *42* (3), 477-483.

111. Halbrook, R.S. and Arenal, C.A. Field Studies Using European Starlings to Establish Causality between PCB Exposure and Reproductive Effects. *Hum. Ecol. Risk Assess.* **2003,** *9*, 121-136; 10.1080/713609855.

112. Tori, G.M. and Peterle, T.J. Effects of PCBs on mourning dove courtship behavior. *Bull. Environ. Contam. Toxicol.* **1983,** *30* (1), 44-49.

113. Palanza, P.; Morellini, F.; Parmigiani, S.; vom Saal, F.S. Prenatal exposure to endocrine disrupting chemicals: effects on behavioral development. *Neurosci. Biobehav. Rev.* **1999,** *23* (7), 1011-1027.

114. Klaassen, M.; Hoye, B.J.; Nolet, B.A.; Buttemer, W.A. Ecophysiology of avian migration in the face of current global hazards. *Philos. Trans. R. Soc. Lond. B. Biol. Sci.* **2012,** *367* (1596), 1719-1732; 10.1098/rstb.2012.0008.

115. US EPA 2008 Method 1668B: Chlorinated Biphenyl Congeners in Water, Soil, Sediment, Biosolids, and Tissue by HRGC/HRMS. Washington (DC): Engineering and Analysis Division, Office of Science and Technology, Office of Water, U.S. Environmental Protection Agency . **2008,** 1-128.

116. Sillett, T.S. and Holmes, R.T. Variation in survivorship of a migratory songbird throughout its annual cycle. *J. Anim. Ecol.* **2002,** *71*, 296-308; 10.1046/j.1365-2656.2002.00599.x.

117. Åkesson, S. and Hedenström, A. How Migrants Get There: Migratory Performance and Orientation. *BioScience* **2007,** *57* (2), 123-133; 10.1641/B570207.

118. Meijer, T.; Möhring, F.J.; Trillmich, F. Annual and Daily Variation in Body Mass and Fat of Starlings *Sturnus vulgaris*. *J. Avian Biol.* **1994,** *25*, 98-104; 10.2307/3677026.

119. Evans, P.R. Autumn movements, moult and measurements of the Lesser Redpoll *Carduelis flammea cabaret*. *Ibis* **1966,** *108* (2), 183-216; 10.1111/j.1474-919X.1966.tb07267.x.

120. Schwab, R.G. Circannian testicular periodicity in the European starling in the absence of photoperiodic change. In *Biochronometry,* Menaker, E.M., Ed.; National Academy of Sciences: Washington, D.C., 1971; pp. 428-447.

121. Lambright, C.; Ostby, J.; Bobseine, K.; Wilson, V.; Hotchkiss, A.K.; Mann, P.C.; Gray, L.E.,Jr. Cellular and molecular mechanisms of action of linuron: an antiandrogenic herbicide that produces reproductive malformations in male rats. *Toxicol. Sci.* **2000,** *56* (2), 389-399.

122. Sughrue, K.M.; Brittingham, M.C.; French, J.B. Endocrine Effects of the Herbicide Linuron on American Goldfinch (*Carduelis tristis*). *Auk* **2008,** *125*, 411-419; http://dx.doi.org/10.1525/auk.2008.06264.

123. Quinn, M.J.,Jr; French, J.B.,Jr; McNabb, F.M.; Ottinger, M.A. The effects of polychlorinated biphenyls (Aroclor 1242) on thyroxine, estradiol, molt, and plumage characteristics in the American kestrel (*Falco sparverius*). *Environ. Toxicol. Chem.* **2002,** *21* (7), 1417-1422; 10.1002/etc.5620210713.

124. DeLeon, S.; Halitschke, R.; Hames, R.S.; Kessler, A.; DeVoogd, T.J.; Dhondt, A.A. The effect of polychlorinated biphenyls on the song of two passerine species. *PLoS One* **2013,** *8* (9), e73471; 10.1371/journal.pone.0073471.

125. McCarty, J.P. and Secord, A.L. Nest-Building Behavior in PCB-Contaminated Tree Swallows. *Auk* **1999,** *116* (1), 55-63.

126. McCarty, J.P. and Secord, A.L. Reproductive ecology of tree swallows (*Tachycineta bicolor*) with high levels of polychlorinated biphenyl contamination. *Environ. Toxicol. Chem.* **1999,** *18*, 1433-1439; 10.1002/etc.5620180713.

127. Perdeck, A.C. Experiment on Orientation of Juvenile Starlings during Spring Migration. *Ardea* **1974,** *62* (3-4), 190-195.

128. Able, K.P. and Bingman, V.P. The Development of Orientation and Navigation Behavior in Birds. *Q. Rev. Biol.* **1987,** *62* (1), 1-29.

129. Mettke-Hofmann, C. and Gwinner, E. Long-term memory for a life on the move. *Proc. Natl. Acad. Sci. U. S. A.* **2003,** *100* (10), 5863-5866; 10.1073/pnas.1037505100.

130. Thorup, K.; Holland, R.A.; Tottrup, A.P.; Wikelski, M. Understanding the Migratory Orientation Program of Birds: Extending Laboratory Studies to Study Free-Flying Migrants in a Natural Setting. *Integr. Comp. Biol.* **2010,** *50* (3), 315-322; 10.1093/icb/icq065.

131. Brewer, A.D.; Diamond, A.W.; Woodsworth, E.J.; Collins, B.T.; Dunn, E.H. Canadian Atlas of Bird Banding. Volume 1: Doves, Cuckoos, and Hummingbirds through Passerines, 1921-1995, second edition [online]. Canadian Wildlife Service Special Publication. Available from Environment Canada through the Internet. **2006**.

132. Macphail, E.M. The role of the avian hippocampus in spatial memory. *Psicológica* **2002,** *23*, 93-108.

133. LaDage, L.D.; Roth, T.C.,2nd; Pravosudov, V.V. Hippocampal neurogenesis is associated with migratory behaviour in adult but not juvenile sparrows (*Zonotrichia leucophrys* ssp.). *Proc. Biol. Sci.* **2011,** *278* (1702), 138-143; 10.1098/rspb.2010.0861.

134. Bingman, V.P. and Able, K.P. Maps in birds: representational mechanisms and neural bases. *Curr. Opin. Neurobiol.* **2002,** *12* (6), 745-750; 10.1016/S0959-4388(02)00375-6.

135. Healy, S.D.; Gwinner, E.; Krebs, J.R. Hippocampal volume in migratory and non-migratory warblers: effects of age and experience. *Behav. Brain Res.* **1996,** *81* (1-2), 61-68; 10.1016/S0166-4328(96)00044-7.

136. Pravosudov, V.V.; Kitaysky, A.S.; Omanska, A. The relationship between migratory behaviour, memory and the hippocampus: an intraspecific comparison. *Proc. Biol. Sci.* **2006,** *273* (1601), 2641-2649; 10.1098/rspb.2006.3624.

137. Gould, E.; Woolley, C.S.; McEwen, B.S. The hippocampal formation: morphological changes induced by thyroid, gonadal and adrenal hormones. *Psychoneuroendocrinology* **1991,** *16* (1-3), 67-84; 10.1016/0306-4530(91)90071-Z.

138. Bingman, V.P. and Jones, T.J. Sun compass-based spatial learning impaired in homing pigeons with hippocampal lesions. *J. Neurosci.* **1994,** *14* (11 Pt 1), 6687-6694.

139. Harrison, X.A.; Blount, J.D.; Inger, R.; Norris, D.R.; Bearhop, S. Carry-over effects as drivers of fitness differences in animals. *J. Anim. Ecol.* **2011,** *80* (1), 4-18; 10.1111/j.1365-2656.2010.01740.x.

140. Newton, I. Can conditions experienced during migration limit the population levels of birds? *Journal of Ornithology* **2006,** *147*, 146-166; 10.1007/s10336-006-0058-4.

141. Armistead, G.L. and Iliff, M.J. The Vagrancy of Gray Kingbird in North America. *North American Birds* **2003,** *57* (2), 148-161.

142. Thorup, K. Vagrancy of Yellow-browed Warbler *Phylloscopus inornatus* and Pallas's Warbler *Ph. proregulus* in north-west Europe: misorientation or great circles? *Ringing & Migration* **1998,** *19*, 7-12; 10.1080/03078698.1998.9674155.

143. Drouillard, K.G. and Norstrom, R.J. Dietary absorption efficiencies and toxicokinetics of polychlorinated biphenyls in ring doves following exposure to aroclor® mixtures. *Environ. Toxicol. Chem.* **2000,** *19*, 2707-2714; 10.1002/etc.5620191114.

144. Muheim, R.; Jenni, L.; Weindler, P. The Orientation Behaviour of Chaffinches, *Fringilla coelebs*, Caught during Active Migratory Flight, in Relation to the Sun. *Ethology* **1999,** *105 (2)*, 97-110; 10.1046/j.1439-0310.1999.00357.x.

145. Harris, M.L. and Elliot, J.E. Effects of Polychlorinated Biphenyls, Dibenzo-p-Dioxins and Dibenzofurans and Polybrominated Diphenyl Ethers in Wild Birds, In *Environmental Contaminants in Biota. Interpreting Tissue Concentrations, Second Edition.* Beyer, W.N. and Meador, J.P., Eds.; CRC Press: 2011; pp. 477-528.

146. Ulfstrand, S. and Södergren, A. Effect of PCB on Nocturnal Activity in Caged Robins, *Erithacus rubecula* L. *Nature* **1971,** *231*, 467-468.

147. Vyas, N.B.; Hill, E.F.; Sauer, J.R.; Kuenzel, W.J. Acephate affects migratory orientation of the white-throated sparrow (*Zonotrichia albicollis*). *Environ. Toxicol. Chem.* **1995,** *14* (11), 1961-1965; 10.1002/etc.5620141118.

148. Clayton, N.S. and Krebs, J.R. Hippocampal growth and attrition in birds affected by experience. *Proc. Natl. Acad. Sci. U. S. A.* **1994,** *91* (16), 7410-7414; 10.1073/pnas.91.16.7410.

149. Bingman, V.P.; Ioale, P.; Casini, G.; Bagnoli, P. The avian hippocampus: evidence for a role in the development of the homing pigeon navigational map. *Behav. Neurosci.* **1990,** *104* (6), 906-911.